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STUDIES ON CHROMOPROTEINS IN JAPANESE NORI, PORPHYRA TENERA

IV. PROPERTIES OF CHROMOPEPTIDES DERIVED FROM PHYCOERYTHRIN BY THE ENZYMIC DIGESTION

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The prosthetic group of phycoerythrin of marine alga was studied previously by Kitasato (*1*) and Lemberg (*2*, *3*, *4*), and has been called "Phycoerythrobilin" since it resembles "Mesobilirhodin" (*4*, *5*), a kind of bile pigment.

It has also been shown that the prosthetic group is firmly bound to the protein moiety and the linkage is not splitted from the protein part under the usual condition of acidic or enzymic hydrolysis. The prosthetic group has however, not yet been isolated in a pure form (*3*, *4*).

The author (*6*) previously obtained the chromopeptides from the peptic hydrolysate of crystalline phycoerythrin of *Porphyra tenera.* Thus chromopeptides in the hydrolysate were extracted with amyl alcohol and purified by column chromatography on Amberlite IRC-50. Sixteen large chromopeptides were isolated by this method. The main component "C_1-Peptide" was composed of about forty amino acid residues and possessed phenylalanine as the N-terminal.

In the present investigation, phycoerythrin was further hydrolyzed with other protease (trypsin, bacterial proteinase—proteinase of *Bacillus subtilis* N′, and *Streptomyces griseus* proteinase) in an attempt to obtain the core chromopeptide. In addition, some properties of chromopeptides will be described.

EXPERIMENTALS

Materials—The crystalline phycoerythrin used in these experiments was prepared as described previously (*7*) from the air-dried Japanese Nori, *Porphyra tenera.* The pepsin and trypsin used were twice crystallized commercial Worthington samples. The crystalline *streptomyces griseus* proteinase used was supplied by the Scientific Research Institute, Ltd. Tokyo Japan. The crystalline proteinase of *Bacillus subtilis* N′ (*8*) used was kindly supplied from the Okunuki's Laboratory, Faculty of Science, Osaka University.

Hydrolysis of C_1-peptide by Trypsin and Proteinase of Bacillus subtilis N′—The both hydrolyses were carried out for 24 hours at pH 7.0 and at 30°. The ratio of enzyme to substrate used was 1 : 30 for trypsin and 1 : 40 for bacterial proteinase. The enzymic hydro-

lysates were acidified and chromopeptide fractions were extracted with butanol. The extracts after removing the butanol, were separated on starch column using phosphate buffer at 7.0 as eluent.

Hydrolysis of Phycoerythrin with Pepsin and Streptomyces griseus Proteinase—In this experiment with *S. griseus* proteinase, a mixture of chromopeptides in the peptic hydrolysis of phycoerythrin was futher hydrolyzed without any purification. Five grams of phycoerythrin were digested with pepsin as in the previous reports (*6*). To the amyl alcohol extract from the acidic hydrolysate, seven volumes of petroleum ether were added and the mixture was allowed to stand overnight. The upper and lower phases were separately dried *in vacuo* below 40° in a current of carbon dioxide. The residue of the upper phase was dissolved in methyl alcohol, the insoluble materials were removed by centrifugation and the supernatant was dried. The residue of the lower phase—amyl alcohol layer—was dissolved in *M*/10 phosphate buffer pH 7.5. To the solution 160 mg. of *S. griseus* proteinase was added and the mixture was stood at 30°, for 24 hours. After the digestion, the solution was brought to pH 1 with hydrochloric acid, and violet colored chromopeptides were extracted with amyl alcohol. To the extract seven volumes of petroleum ether were added and the mixture was allowed to stand overnight. The upper and lower phases were separately dried as described above. The upper phase (petroleum ether-amyl alcohol layer II) is purple and the lower phase is violet.

Amino Acid Analysis of Chromopeptides

Hydrolysis of Chromopeptides—Chromopeptide fractions were hydrolyzed in a sealed tube with 5.7 *N* HCl at 105–110° for 24 or 36 hours. The hydrolysate was concentrated to dryness *in vacuo* to remove hydrochloric acid.

Qualitative Analysis—Hydrolysates were separated by two-dimensional paper chromatography using *n*-butanol-acetic acid-water (4 : 1 : 1 v/v) as the solvent for the first dimention, and 80 per cent phenol-NH_3 or *n*-butanol-methylethylketone-water-17 *N* ammonia (5 : 3 : 1 : 1 v/v) for the second dimension. The spots on paper chromatograms were located by spraying with ninhydrin (*23*, *24*).

Quantitative Analysis—For the quantitative determination of amino acids, Moore and Stein method (*9*) or paper chromatography of dinitrophenyl (DNP) amino acid (*10*, *11*) were used. Cystine was determined by the hydrazinolysis according to Akabori and Fujiwara (*12*). The leucine and isoleucine in P-12a peptide were determined by bioassay in addition to DNP method (*10*, *11*).

Identification of Terminal Groups

N-terminal Group of Chromopeptides—Chromopeptides were dinitrophenylated and hydrolyzed by Sanger method and determined spectrophotometrically, after the paper chromatographic separation (*10*, *11*).

C-terminal Group of Chromopeptides—C-terminal amino acid was determined by hydrazinolysis according to Akabori *et al.* (*15*).

RESULTS

Hydrolysis of C_1-peptide by Trypsin and by B. subtilis N′ Proteinase—Since the C_1-peptide obtained from phycoerythrin with pepsin was a rather large molecule (*6*), it was further hydrolyzed by trypsin and by *B, subtilis* N′ proteinase.

After the enzymic hydrolysis, the extracts with *n*-butanol were separated

on starch columns as described above. Eight colored zones were obtained from the tryptic digest, and six zones from the *B. subtilis* N′ proteinase digest. These zones were red, violet, yellow, brown and green in color, as shown in Fig. 1.

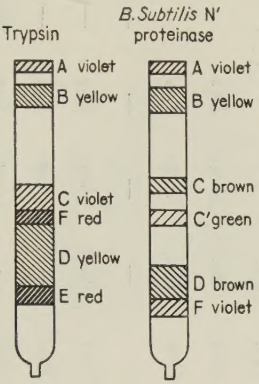


FIG. 1. Chromatogram of chromopeptides derived from C_1-peptide with trypsin and with *B. subtilis* N′ proteinase.

Chromatography was carried out on a 2.0× 40 cm. column of starch. 0.1 *M* phosphate buffer at pH 7.0 was used as the eluent.

These chromopeptides were all soluble in water except for tryptic-A, -B and -C′, *B. subtilis* N′ proteolytic-A and C peptides. These water insoluble peptides were soluble in alcohol. Tryptic-C′ was prepared to extract from the zone of tryptic-C with alcohol after the extraction with water.

On the hydrolysis (5.7 *N* HCl at 105–110°, for 24 hours), these chromopeptides produced eight amino acids, *i.e.*, aspartic and glutamic acids, glycine, alanine, valine, leucine and isoleucine commonly, which were identified by paper chromatography (Table 1). The results of the quantitative determination of amino acid constituents as the dinitrophenyl amino acid (*10*, *11*) indicated that tryptic-A contained 19 amino acid residues; (Asp and Glu)$_3$, Ser$_2$, Gly$_2$, Ala$_4$, Val$_2$, (Leu and Ileu)$_3$, Phe$_1$ and Lys$_2$, tryptic-C had 14 amino acid residues; (Asp and Glu)$_3$, Ser$_2$, Gly$_1$, Ala$_2$, Val$_1$, (Leu and Ileu)$_3$, Phe$_1$ and Lys$_1$, and *B. subtilis* N′ proteolytic-C′ had ten amino acid residues; (Asp and Glu)$_3$, Ser$_1$, Gly$_1$, Ala$_1$, (Leu and Ileu)$_2$, Phe$_1$ and Lys$_1$. No N-terminal amino acid could be detected by Sanger's method in the peptides, tryptic-A, C and C′, and *B. subtilis* N′ proteolytic-C and C′.

Further Hydrolysis of the Tryptic-A Peptide—The tryptic-A peptide obtained above, was subjected to the partial hydrolysis with concentrated hydrochloric acid at 37° for 48 hours and chromopeptide fragment was extracted from a weakly acid solution with amyl alcohol. In this case a part of the chromopeptide fragment was remained in the acid layer. The chromopeptide in the amyl alcohol-extract was dinitrophenylated and the product was extracted

TABLE I

Amino Acid Constituent of Chromopeptides Obtained from C_1 Peptide by Enzymic Hydrolysis[1)]

Enzyme	Peptide	Amino Acid												
B. subtilis N′ proteinase	C	—Asp	Glu	Gly	Ser	Ala	Val	Leu	Ileu	(Phe)				
	C′	—Asp	Glu	Gly	Ser	Ala	Val	Leu	Ileu	(Phe)	(Arg)	Lys		
trypsin	A	—Asp	Glu	Gly	Ser	Ala	Val	Leu	Ileu	(Phe)	(Arg)	Lys		
	B	—Asp	Clu	Gly	Ser	Ala	Val	Leus		—	—	—	—	Tyr
	C[2)]	—Asp	Glu	Gly	Ser	Ala	Val	Leu	Ileu	(Phe)	(Arg)	Lys	—	—
	C′[3)]	—Asp	Glu	Gly	(Ser)	Ala	Val	Leu	Ileu	—	(Arg)	Lys	—	Tyr
	D	—Asp	Glu	Gly	Ser	Ala	Val	Leu	Ileu	—	(Arg)	Lys	His	Tyr
	F	—Asp	Glu	—	Ser	A[a	Val	Leu	Ileu	—	Arg	—	—	—

( ) Unidentified.

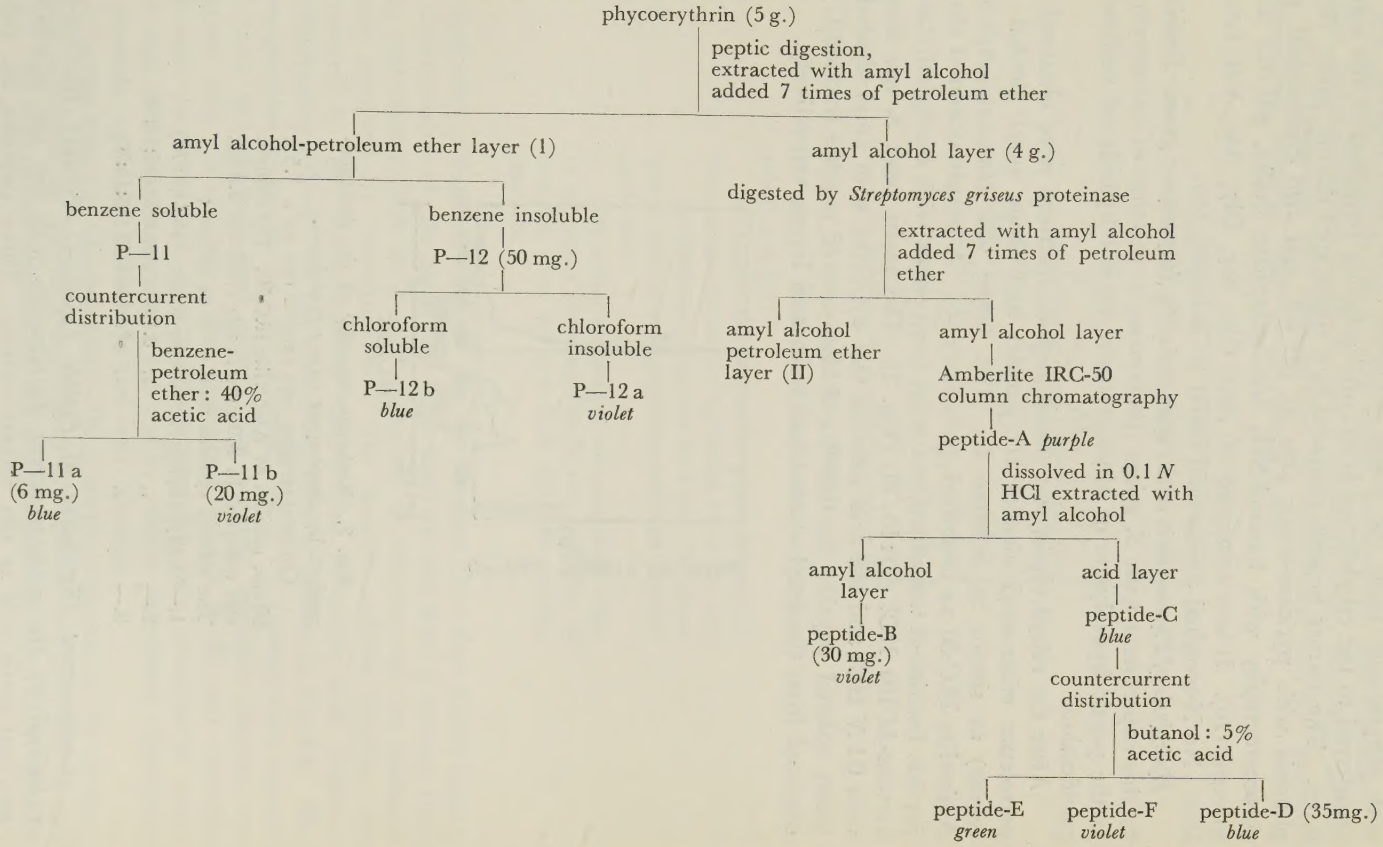
1) detected by ninhydrin method on paper chromatography.

2) water soluble fraction of the zone of tryptic-C

3) water insoluble fraction of the zone of tryptic-C

SCHEME I

Preparation of Chromopeptides from Phycoerythrin



with ethylacetate from the acidic solution. One DNP-peptide was thus transferred to the ethylacetate layer, while another remained in the aqueous layer. The former peptide migrated as a single spot during paper electrophoresis with pyridine-acetic acid at pH 6.0, and two dimentional paper chromatography with butanol-NH_3 and phosphate buffer, pH 6.5 as the solvents (*10*). It was composed of Asp, Glu, Ser, Gly, Ala, Val, Leu and Ileu, No N-terminal amino acid could be detected, too.

Hydrolysis of Phycoerythrin with Pepsin and with Streptomyces griseus Proteinase—In this experiment with *S. griseus* proteinase, a mixture of chromopeptides in the peptic hydrolysate of phycoerythrin were rehydrolyzed without any purification.

After the rehydrolysis, two chromopeptide fractions were obtained from petroleum ether-amyl alcohol II (purple) and from amyl alcohol layer (violet) as shown in Scheme I. The latter was chromatographed on Amberlite IRC-50 as described previously (*6*). A purple coloured chromopeptide fraction-A was eluted as a main component with methylethylketone-acetone-*M*/100 HCl (15 : 15 : 70) (Fig. 2). The main peak A was dissolved with 0.1 *N* HCl and the acid solution was extracted with amyl alcohol. A violet colored peptide fraction-B and blue colored peptide fraction-C were obtained from the amyl alcohol and from acid layers, respectively.

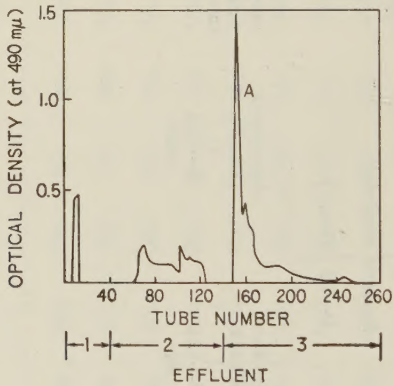


FIG. 2. Rechromatography of the digestion-product by *Streptomyces griseus* proteinase.

Chromatography was carried out on a 1.5× 60 cm. column of Amberlite IRC-50. The effluent was collected in 260 tubes, each receiving 2.0 ml. The solvent systems used were as follows;

1.—Methylethylketone-acetone-water (15 : 15 : 70)
2.— ,, ,, 0.1*M* acetic acid (15:15:70)
3.— ,, ,, 0.01*M* HCl (15:15:70)

Countercurrent Distribution of Peptide Fraction-C—Peptide fraction-C was examined by the countercurrent distribution with the use of *n*-butanol-5 per cent acetic acid as the solvent system. Three peptides, *i.e.*, peptide-D

(blue), E (green), F (violet) were obtained as shown in Fig. 3-a. Peptide-D was hydrophilic, while E and F were rather hydrophobic and unstable.

The chromopeptides-B, D, E and F obtained from the amyl alcohol layer were hygroscopic and were soluble in alcohol and acid but insoluble in chloroform, benzene, ether or petroleum ether.

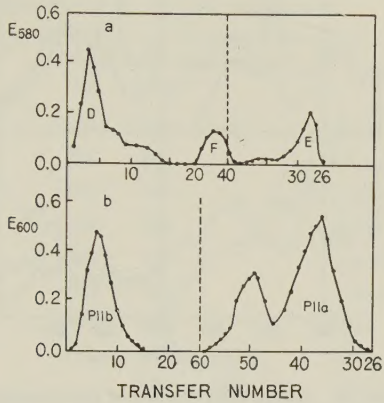


FIG. 3. Countercurrent distribution of peptide-C and P-11.

a—Distribution patterns for peptide fraction-C. The solvent system was *n*-butanol-5% acetic acid.

b—Distribution patterns for P-11. The solvent system was benzene petroleum ether (30 : 45)-40% acetic acid.

The concentrations plotted, correspond either to the upper phase solution in cells 0–26 after 40 transfers (Fig. 3a) and 0–26 after 60 transfers (Fig. 3b).

Purification of Pigments Obtained from the Petroleum Ether-Amyl Alcohol Layer—The dried residue of the petroleum ether-amyl alcohol layer I derived from peptic hydrolysate (Scheme I.) was dissolved in benzene and the insoluble fraction removed by centrifugation. The benzene soluble fraction was fractionated by the countercurrent distribution technique with benzene-petroleum ether (35 : 40)—40 per cent acetic acid as shown in Fig. 3-b. The chromopeptides obtained, *i.e.*, P-11a (blue) and P-11b (violet) were crystallized from benzene-petroleum ether. The benzene insoluble fraction was extracted with chloroform. The chloroform soluble P-12b and insoluble P-12a were thus obtained. These chromopeptides were insoluble in water, dilute acid and petroleum ether, but readily soluble in acetone chloroform and alcohols, especially in methyl and ethyl alcohol.

Absorption Spectra of Chromopeptides—The absorption spectra of the chromopeptides obtained by the various methods are summarized in Table II and Fig. 4. The absorption maxima of the chromopeptides were all at 490–500

mμ and 550–610 mμ, except for that of peptide-E with maxima at 558 and 598 mμ. There are two types of chromopeptides which possessed different absorption spectra from each other, thus B, D and P-12a had maxima

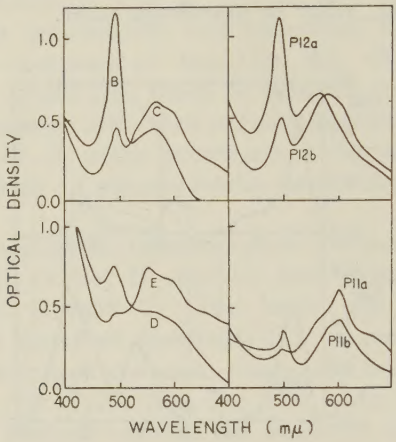


FIG. 4. Absorption spectra of chromopeptides derived from phycoerythrin.

between 490–500 mμ higher than that between 550–610 mμ, while C, E, P-11a and P-11b maxima between 550–610 mμ higher than that between 490–500 mμ. The peptides with the former maxima were purple or violet and those with the latter were blue. The peptide P-11a differed from other chromopeptides at the ultra-violet absorption, showing three peaks at 271, 332 and 373 mμ. It is of interest that the similar ultra-violet absorption is found in that of phycoerythrin as shown in Fig. 5.

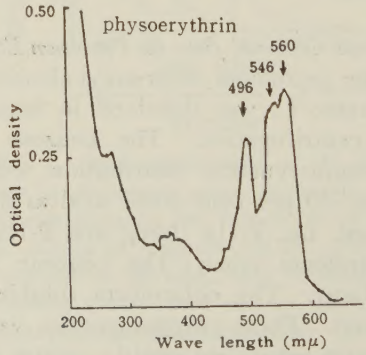


FIG. 5. Absorption spectra of phycoerythrin and P-lla.

Amino Acid Analysis of the Chromopeptides Obtained from the Petroleum Ether-Amyl Alcohol Layer and the Digestion Product of S. griseus Proteinase—Chromopepti-

des were hydrolyzed for 24, 36 and 72 hours and the best result was obtained with 36 hours for hydrolysis. Therefore all peptides were subsequently hydrolyzed with 5.7 *N* HCl at 105–110° for 36 hours in this experiment.

TABLE II

Absorption Spectra of Chromopeptides Derived from Phycoerythrin by Enzymic Digestion[1)]

Peptide	Absorption maxima (mμ)			Color
P—11 a	(496)	610	658	blue
P—11 b	496	600		violet
P—12 a	493	568		violet
P—12 b	(498)	580		blue
A	493	570		purple
B	493	566		violet
C	496	568		blue
D	495	574		blue
E		558	598	green

( ) weak absorption.
1) Ethyl alcohol was used as solvent.

The results of amino acid composition of chromopeptides are summarized in Table III. Although seventeen amino acids are present in phycoerythrin, eight *i.e.*, Asp, Glu, Ser, Gly, Ala, Val, Leu and Ileu are commonly found in the chromopeptides. The basic amino acid, arginine present in peptide-B was not found in the other peptides. Proline was present only in P-11b and P-12a. The benzene soluble P-11a and P-11b contained less amino acids. The former especially had only trace amino acids consisting of 1.76 per cent of amino acid-nitrogen of the total nitrogen. Leucine and isoleucine

TABLE IV

Elemental Analysis and Minimum Molecular Weight

	C	H	N	S	P[1)]	Ash	Fe[2)]	S : N	MW[3)]
Phycoerythrin	50.80	7.99	15.13	1.72	0.61	1.06	0.45		
C_1-peptide	44.26	5.25	14.53	2.36		3.91			
B	51.78	6.56	5.34	1.30		11.10		1 : 9	2,460
D	34.35	4.46	6.87	none					
P-11a	53.37	6.61	5.20	0.67		6.02	0.78	1 : 18	4,795
P-11b	50.60	6.94	5.89	1.65		8.60		1 : 9	1,945
P-12a	38.86 39.04	4.82 4.78	6.04	1.45 1.49	none	7.12 7.29		1 : 9	2,145

1) Fiske-Subarrow's method (*21*) 2) Thiocyanate's method (*22*)
3) Calculated for 1.0 mole of sulfur in each peptide chain.

TABLE III

Amino Acid Composition of Chromopeptides

	P—11a[c]	P—11b[b]		P—11 a[c]		B[b]		D[c]
		Mol. of amino acid per 10 g. of peptide	Mol. of amino acid per 1.0 mol. of sulfur	Mol. of amino acid per 10 g. of peptide	Mol. of amino acid per 1.0 mol. of sulfur	Mol. of amino acid per 10 g. of peptide	Mol. of amino acid per 1.0 mol. of sulfur	Mol. of amino acid per 10 g. of peptide
aspartic acid	+	0.10	0.019	1.65	0.368	1.18	0.288	0.92
glutamic acid	+	0.17	0.033	0.95	0.211	0.53	0.129	
serine	++	0.07	0.013	0.51	0.113	1.62	0.385	0.68
glycine		0.30	0.058	0.88	0.196	1.21	0.296	1.84
alanine	+	0.25	0.049	2.15	0.479	1.40	0.342	0.97
valine				1.57	0.350	1.40	0.342	
leucine	++	1.08	0.212	3.78[a] } 4.72	0.842[a] } 1.050	0.57	0.138	1.53
isoleucine				2.01[a]	0.532[a]	0.82	0.200	
proline		1.07	0.211	0.76	0.169	—	—	—
arginine		?		?	?	1.10	0.268	—
mol. of total N per 10 g. peptide	37.2	42.7		43.1		38.2		48.1
mol. of sulfur per 10 g peptide	2.1	5.2		4.5		4.1		0.0
$\frac{\text{amino acid-N}}{\text{total-N}}$ 100	1.76	7.14		42.3		25.4		10.5

a) bioassay *b)* Stein and Moore's method. *c)* DNP method.

contents were rather higher in P-11a, P-11b and P-12a. In P-12a, leucine and isoleucine were determined by bioassay and by the DNP method. The results from these methods were in good agreement. Leucine and isoleucine in P-12a were confirmed to be the L-forms by bioassay.

No N-terminal amino acid could be determined by Sanger's method (*14*) in any chromopeptides. On hydrazinolysis (*15*) of peptide-A although a DNP derivative was obtained as described in the previous report (*6*), no C-terminal amino acid could be found.

Elemental Analysis—The results of the elemental analysis of the chromopeptides are summarized in Table IV. The per cent of nitrogen of all of the chromopeptides obtained from petroleum ether-amyl alcohol layer and from redigests with *S. griseus* proteinase is as low as 5–6 per cent. As shown in Table IV, all of the chromopeptides contain sulfur at 0.7 to 1.5 per cent, except peptide-D. Phycoerythrin and C_1-peptide also have non-amino acid sulfur as shown in the following data:

	Phycoerythrin (%)	C_1-peptide (%)	Method
total-S	1.72	2.36	strong phosphoric acid (*16*)
methionine-S	0.32	—	performic acid (*17*)
half cystine-S	0.59	1.04	hydrazinolysis (*12*)
unidentified-S	0.79	1.32	

There may be some sulfur containing groups in the neighbourhood of the chromophore group. The minimum molecular weight and molar ratio of the elements per 1.0 mole of sulfur in each peptide chain as calculated from the results of elemental analyses, are as shown in Table IV. The ratio of nitrogen to sulfur was 9 : 1 or 18 : 1 in these chromopeptides.

Sugar Content of the Chromopeptides—The presence of sugar was suggested by the high oxygen content found by elemental analysis and from the fact that phycoerythrin was a glucoprotein as shown in the previous report* (*7*). Sugar contents of P-11a, P-11b and P-12a were therefore measured by orcinol-sulfuric acid method (*18*). As shown in Table V, 13.01, 7.79 and 3.89 per cent glucose values were found, respectively.

TABLE V

Suger Content of Chromopeptides
(glucose value[1])

Peptide	Hydrolysis	Sugar (%)
P—11 a	3 *N* HCl 4 hrs	13.01
P—11 b	3 *N* HCl 4 hrs	7.79
P—12 a	2 *N* HCl 2 hrs	3.89

1) Orcinol-sulfuric acid method (*17*).

Infra-red Absorption Spectra—Infra-red absorption spectra of P-11b, 12a

* Unpublished data.

and peptide-B and -D are shown in Fig. 6. At the region between wave number 650-1000 and 3000-3500 cm^{-1}, there is much difference in the spectra P-12b especially, differing from the other three.

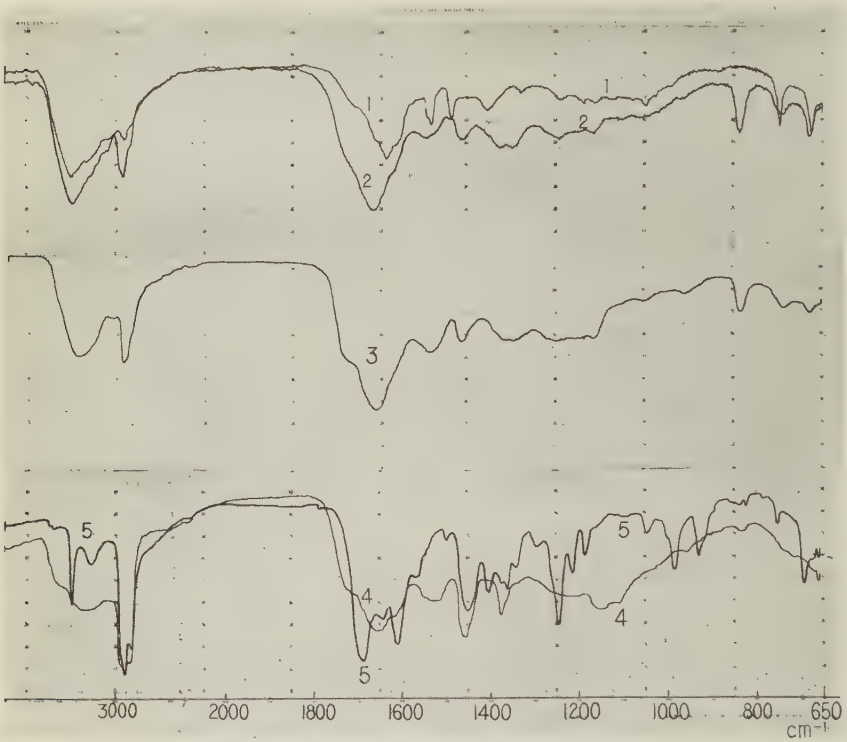


Fig. 6. Infra-red absorption spectra of chromopeptides.
1; D 2; B 3; P-11 b 4; P-12 a 5; Bilirubin

DISCUSSION

The chromopeptides phycoerythrin obtained by the proteolytic digestions with pepsin, trypsin, *B. subtilis* N′ proteinase and *S. griseus* proteinase are hydrophobic and generally soluble in organic solvents, especially, in methyl and ethyl alcohol.

The rehydrolysis with trypsin and *B. subtilis* N′ proteinase of a higher peptide from the peptic digest resulted in chromopeptides containing ten amino acids, Asp, Glu, Ser, Gly, Ala, Val, Leu, Ileu, Phe and Lys. Rehydrolysis with *S. griseus* proteinase resulted in chromopeptides containing Asp, Glu, Ser, Gly, Ala, Val, Leu and Ileu but without phenylalanine and lysine. After the peptic digestion, the petroleum ether-amyl alcohol layer also contained chromopeptides with the same amino acids as in the peptides obtained by hydrolysis with *S. griseus* proteinase. However these peptide had high content of leucine and isoleucine. From the partial acid hydrolysis

of tryptic-A, a chromopeptide was also obtained having the same amino acids. The results suggest that there is a core chromopeptide composed of Asp, Glu, Ser, Gly, Ala, Leu and Ileu which results from limited proteolysis.

As the amino acids in the sugar containing peptides are decomposed during acid hydrolysis, the true amino acid content would be somewhat higher than that found in the present data.

The results of elemental analysis are shown in Table IV. Peptide-B and -D and P-11a, -11b and -12a have very low nitrogen contents (5–6 per cent), as compared with that of mesobilirhodin (9.6 per cent). The author has shown that the discrepancy is due to the presence of sugars in the chromopeptides. The sugars contents were 13.01, 7.79 and 3.89 per cent as glucose for P-11a, -11b and -12a, respectively. No sugar alcohol (*20*) could be found on paper chromatography.

As it will be reported in detail in the proceeding paper (*25*), chromopeptide P-12a contain three sugars, *i.e.*, galactose, arabinose and xylose at the amount of 0.87, 0.56 and 0.31 per cent and 0.85 per cent of sulfur in the sugar fraction. Chromopeptide P-12a was confirmed to be a glucopeptide.

There are very high ash contents in the chromopeptides as shown in Table IV, although they are soluble in organic solvents. It is suggested from the two points, the sulfur combined to sugar part with the form of sulfate.

With the exception of peptide-D, the chromopeptides all contained non-amino acid sulfur. The ratio of nitrogen to sulfur was 9 : 1 or 18 : 1 in the chromopeptides. This seems to indicate the presence of some group containing sulfur and nitrogen near the chromophores.

No N-terminal amino acid could be determined by the DNP method in any chromopeptide but the C_1-peptide. No C-terminal amino acid could be determined in the C_1-peptide, or the tryptic or *B. Subtilis* N′ proteolytic hydrolysates or peptide-A by hydrazinolysis either.

It is concluded from the present work that these chromopeptides have a peculiar structure.

SUMMARY

1. Various chromopeptides soluble in organic solvents, were obtained from the peptic, tryptic, *B. subtilis* N′ and *S. griseus* proteolytic digests of phycoerythrin of *Porphyra tenera.*

2. Absorption maxima of these chromopeptides were from 490 to 600 mμ.

3. The core chromopeptides of phycoerythrin containing Asp, Glu, Ser, Gly, Ala, Val, Leu and Ileu could be obtained by the proteolysis.

4. Most of the chromopeptides had a low nitrogen content (5–6 per cent) and about 0.6 to 1.5 per cent of sulfur.

5. The sugar contents of the chromopeptides, P-11a, -11b and -12a were 13.01, 7.79 and 3.89 per cent (glucose values) respectively. Galactose, arabinose and xylose were found in P-12a.

The author wishes to express her sincere gratitude to Prof. S. Akabori for his constant interest and encouragement throughout this investigation. She also wishes to thank Prof. K. Okunuki and Dr. H. Matsubara, Faculty of Science, Osaka University for supplying crystalline *Bacillus subutilis* proteinase, and Miss. T. Kawano, Osaka College of Pharmacy for bioassay.

REFERENCES

(*1*) Kitasato, Z., *Acta phytochim.*, **2**, 274 (1925)
(*2*) Lemberg, R., *Ann. Chem.*, **461**, 46 (1929)
(*3*) Lemberg, R., *ibid.*, **477**, 195 (1930)
(*4*) Lemberg, R., *ibid.*, **505**, 151 (1933)
(*5*) Siedel, W., *Z. physiol. Chem.*, **237**, 8 (1935)
(*6*) Fujiwara, T., *J. Biochem.*, **44**, 723 (1957)
(*7*) Fujiwara, T., *ibid.*, **42**, 411 (1955)
(*8*) Hagihara, B., *Ann. Rep. Scient. Works Fac. Sci. Osaka Univ.*, **2**, 35 (1954)
(*9*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211**, 893 (1954)
(*10*) Levy, A. L., *Nature*, **174**, 126 (1954)
(*11*) Koch, G., and Weided, W., *Z. physiol. Chem.*, **303**, 213 (1956)
(*12*) Akaboli, S., and Fujiwara, T., *Bull. soc. chim. biol.*, **40**, 1983 (1958)
(*13*) Fujiwara, T., *J. Biochem.*, **43**, 195 (1956)
(*14*) Sanger, F., and Thompson, A. R., *Biochem. J.*, **53**, 353 (1953)
(*15*) Akabori, S, Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Tsugita, A., Sugae, K., and Matsushima, T., *Bull. Chem. Soc. Japan*, **29**, 507 (1956)
(*16*) Kiba, T., Akaza, I., Kishi, N., and Taki, S., *Bull. Chem. Soc. Japan*, **30**, 482 (1957)
(*17*) Scharam, E., Moore, S., and Bigwood, E. J., *Biochem. J.*, **57**, 33 (1954)
(*18*) Sørensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933)
(*19*) ÓhEocha, C., *Arch. Biochem. Biophys.*, **73**, 207 (1958)
(*20*) Hackman, R. H., and Trikojus, V. M., *Biochem. J.*, **51**, 653 (1952)
(*21*) Fiske, C. H., and Subarrow, Y., *J. Biol. Chem.*, **66**, 375 (1929)
(*22*) Kitson, R. E., *Anal. Chem.*, **22**, 664 (1950)
(*23*) Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944)
(*24*) Wolfe, M., *Biochim. et Biophys. Acta*, **23**, 186 (1957)
(*25*) Fujiwara, T., in press

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METABOLISM OF D-GLUCOSAMINE

IV. ON THE NATURE OF GLUCOSAMINE OXIDATION BY PSEUDOMONAS FLUORESCENS*

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(Received for publication, February 2, 1960)

About the metabolism of D-glucosamine (GA) there have been made several progresses which are on deamination to D-fructose (*1–6*), isomerization to D-galactosamine (CA) (*7*), acetylation to N-acetylglucosamine (*8–11*) followed by further conversion through its uridinediphospho derivative (*12–14*) or by oxidation to N-acetylglucosaminic acid (*15*) and oxidation to glucosaminic acid (GAA) (*16*). The enzymic reactions of the former three pathways have been studied in several laboratories, but about the last one which was found in the extract of *Pseudomonas fluorescens* (*Ps. fluor.*) there has been no report so far. Since it was reported (*17–18*) recently that GAA was deaminated to 2-keto-3-deoxygluconic acid, the latter being decomposed to pyruvic acid and glyceraldehyde phosphate, the GAA formation seems to have had a general biological meaning though GAA has not yet been found in living organisms.

In this paper, the author reports that the activity in *Ps. fluor.* extract for GA oxidation is of some particulate-bound nature and GA oxidation (dehydrogenation) is connected to oxygen through some cytochrome system, that is, GAA formation is carried out by a cytochrome-linked dehydrogenase, not by an oxidase.

EXPERIMENTAL

Materials

D-*Galactosamine* (*CA*)—Prepared from chondroitinsulfuric acid (*19*). It showed one spot on the paperchromatogram (solvent system; pyridine-ethylacetate-water-acetic acid (5 : 5 : 3 : 1) (*20*), *Rfs*; CA 0.34, GA 0.39). Analysis of N (Found 6.27%, Calc. for $C_6H_{13}O_5$ NHCl 6.50%).

Cultivation of bacterium—Same as in the report II of this series (*16*).

Analytical Methods

Centrifugal—Hitachi preparative centrifugal apparatus was used.

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* Reported at the 11th Symposium on Enzyme Chemistry (Kyoto University) in July (1959) by Imanaga, Y., Yoshifuji, K., Momose, N., Kamai, K. and Matsushima, Y..

Spectrophotometrical—Hitachi EPU-2 type spectrophotometer was used. The spectra of particulate suspension were observed by opal-glass method.

Manometrical—All values obtained in Warburg's method were subtracted by control values which contained no substrate.

RESULTS

1. Centrifugal Fractionation of the Cell Disintegrates—The extract which had been used in the preceding paper was the supernatant of the cell disintegrates at 20,000 r.p.m. (ca. 27,000×*g*) for 20 minutes and was not quite transparent (S_{20} in Scheme 1). When this extract was further centrifuged at 35,000 r.p.m. (ca. 100,000×*g*) for 40 minutes, a clear supernatant (S_{35}) and reddish precipitate (R_{35}) were obtained. S_{35} had no activity toward GA and R_{35} suspension in phosphate buffer (PB) had strong activity instead (Table 1). These facts showed that GA oxidizing activity was localized in the reddish particulate fraction (R_{35}).

Besides, it was found that R_{35} powerfully reduced 2,6-dichlorophenol-indophenol (CPIP) anaerobically in the presence of GA.

TABLE I

Localization of GA Oxidizing Activity

Enzymic fraction	Oxygen consumed (μl./60 min.)
(A)	65
(B)	0
(C)	80

(A): S_{20}, dialyzed for 2 days at 0°.

(B): S_{35}, obtained from (A).

(C): Re-suspension in *M*/15 PB (pH 7.3) of R_{35} obtained from (A).

Contents: Enzymic fraction 2.0 ml. ((C) contained about two fold amounts of particulates in A)), GA 0.4 ml. (40 μM), *M*/15 PB (pH 7.0) 1.4 ml., 10 per cent KOH 0.2 ml. (center well). Total 4.0 ml. At 30°.

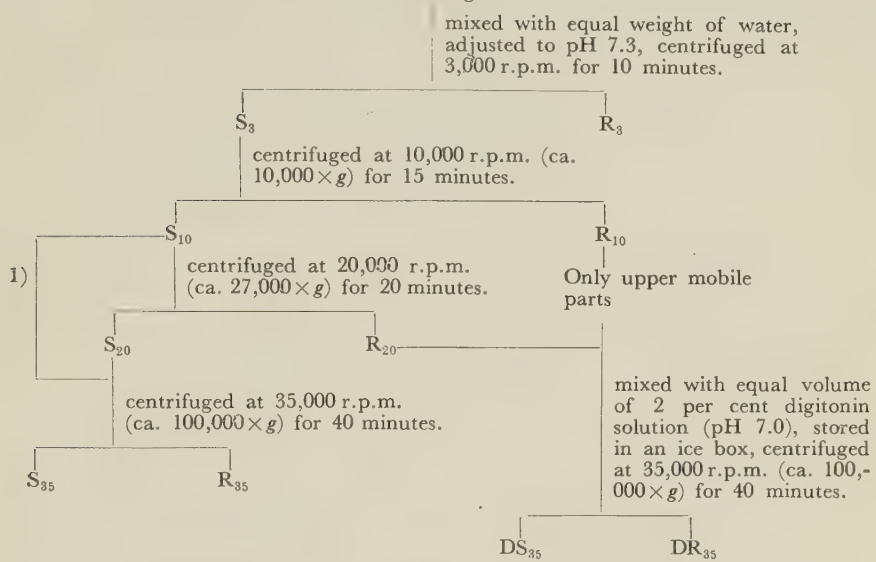
Scheme 1 shows the process of the centrifugal fractionation used in this paper.

2. Substrate Specificity of R_{35}—As shown in Fig. 1, R_{35} consumed one atom of oxygen per molecule of GA or glucose, but it had almost no activity toward CA, galactose and fructose. Further, it could not oxidize N-acetylglucosamine, N-acetylglucosaminol, glucosaminol and lactose.

3. Solubilization from R_{35} of the "GA-dehydrogenase"—When R_{35} was treated with 2 per cent digitonin solution in an ice box and then the mixture

SCHEME 1

Centrifugal Fractionation of the Disintegrates of Ps. fluor.



S: Supernatant, R: Residue, D: Digitonin-treated

1) For preparation of R_{35}, it was preferable to centrifuge S_{10} directly at 35,000 r.p.m.

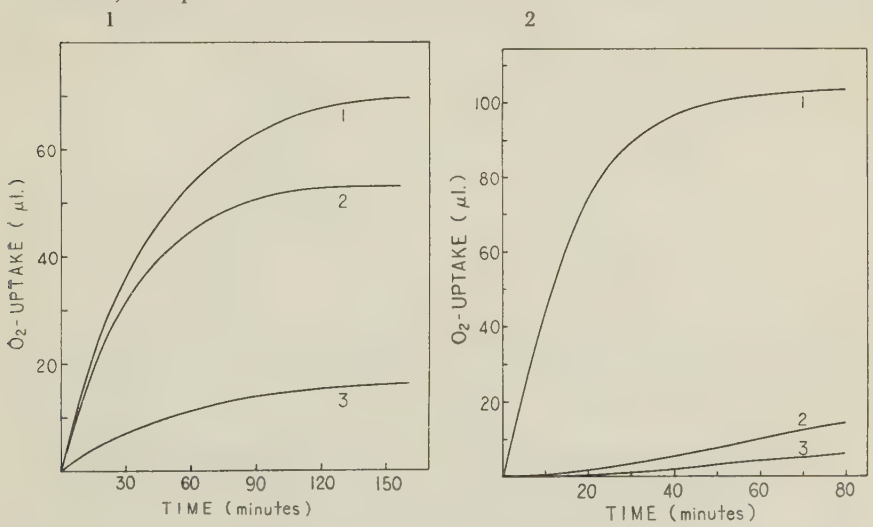


FIG. 1. Activity of R_{35} toward several sugars.

Contents: 1: R_{35} suspension 1.0 ml., substrate (Curve 1: GA 0.2 ml. (5 μM)+CA 0.2 ml. (20 μM), Curve 2: GA 0.2 ml. (5 μM), Curve 3: CA 0.2 ml. (20 μM)), M/15 PB (pH 7.0) 2.4 ml., water (Curve 2: 0.2 ml., Curve 3: 0.2 ml.), 10 per cent KOH 0.2 ml. (center well). Total 4.0 ml. At 30°.

2: substrate 0.4 ml. (10 μM) (Curve 1: glucose, Curve 2: galactose, Curve 3: fructose). Others were the same as in Fig. 1–1.

was centrifuged at 35,000 r.p.m. for 40 minutes, a clear reddish supernatant was obtained. Whereas this supernatant (DS_{35}) showed almost no oxygen consumption for GA, it had powerful activity of reducing CPIP in the presence of the substrate (Table II).

TABLE II

Digitonin Treatment of R_{35}

	Digitonin-treated (DS_{35})		Water-extracted[1]	
	Main	Control	Main	Control
GA (75 μM/ml.)	0.2 ml.	— ml.	0.2	— ml.
CPIP (1 μM/ml.)	0.2	0.2	0.2	0.2
Enzyme fraction	0.5	0.4	0.5	0.5
M/15 PB (pH 7.3)	1.1	1.3	1.1	1.3
Time required for decolorization at 30°.	1 min.	35 min.	18 min.	35 min.

1) Water-extracted : R_{35} was treated with water (pH 7.0) in place of 2 per cent digitonin solution.

When air was introduced into the Thunberg tube which had contained the decolorized reaction mixture, the color of CPIP was soon restored. It

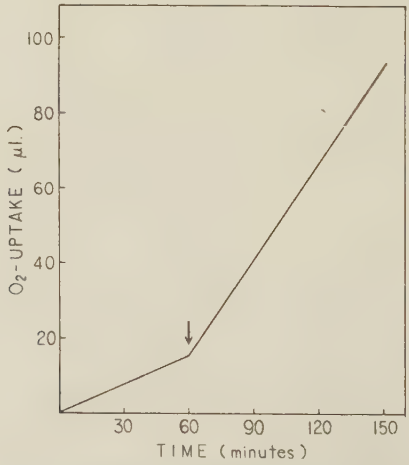


FIG. 2. Effect of CPIP on the oxidation by DS_{35} of GA.

Contents : DS_{35} 1.0 ml., GA 0.4 ml. (30 μM), *M*/15 PB (pH 7.0) 2.0 ml., 10% KOH 0.2 ml. (center well), 0.4 ml. of CPIP (0.4 μM) was added at the point (↓). Total 4.0 ml. At 30°.

seemed from this observation that DS_{35} had the activity of oxidizing reduced CPIP too. It may be postulated that dehydrogenation by DS_{35} of GA could be connected to oxygen through the reduction and re-oxidation of CPIP. In fact, the oxygen consumption by DS_{35} using GA as the substrate was markedly accelerated by the addition of CPIP (Fig. 2).

DS_{35} reduced $K_3[Fe(CN)_6]$ in the presence of GA (Fig. 3), but it had no reducing activity toward triphenyltetrazolium chloride.

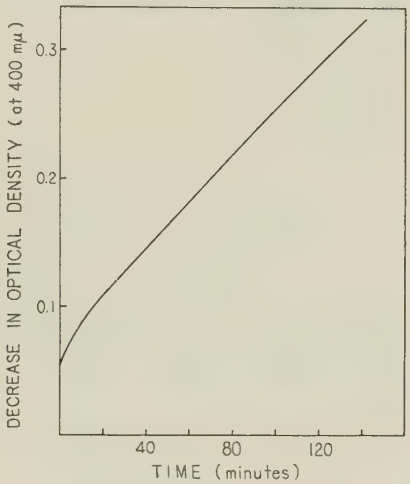


FIG. 3. Reduction by DS_{35} of $K_3[Fe(CN)_6]$ in the presence of GA.

Contents: DS_{35} (dialyzed) 1.0 ml., GA 0.2 ml. (15 μM), $M/100$ $K_3[Fe(CN)_6]$ 0.4 ml., $M/15$ PB (pH 7.3) 1.7 ml. Control was subtracted which contained 0.4 ml. of PB in place of GA. Another control which lacked DS_{35} showed no change in its optical density. At 22.5°.

It is conceivable that the oxidation by R_{35} of GA was carried out by a dehydrogenase (GA dehydrogenase) which could be extracted into DS_{35} and the connection to molecular oxygen of this dehydrogenation reaction was destroyed by the digitonin treatment.

4. Participation of Cytochrome System in the Oxidation by R_{35} of GA:

a. Spectrographycal Studies—Both R_{35} and DS_{35} were spectrophotometrically shown to contain cytochrome(s). As shown in Fig. 4, DS_{35} had its absorption maxima at 415, 520 and 550 mμ and there was almost no change in its spectrum by the addition of GA. On the contrary, R_{35} had only one absorption maximum at 410 mμ and by the addition of GA the shift of absorption maximum (410→420 mμ) was observed and further two new maxima at 530 and 558 mμ appeared, though these were weak. This spectrographycal change was in good coincidence with the results observed

when R_{35} had been chemically oxidized by $K_3[Fe(CN)_6]$ and then reduced by $Na_2S_2O_4$ (Fig. 4, 5, 6).

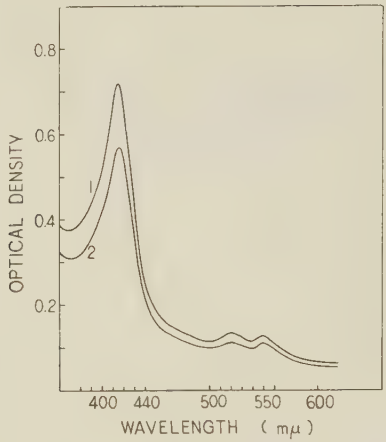


Fig. 4. Absorption spectra of DS_{35}
Contents: Curve 1: DS_{35}. Curve 2: 40 minutes after replacing 0.8 ml. in Curve 1 by 0.8 ml. of GA (60 μM). pH 7.0. At 25°.

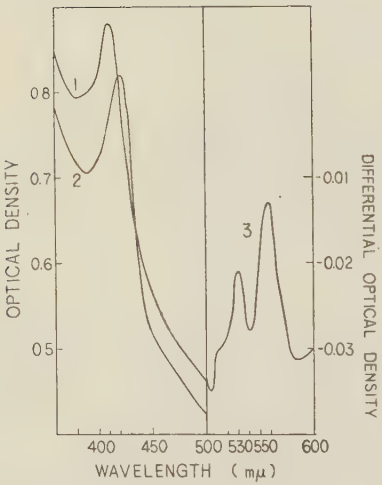


Fig. 5. Absorption spectra of R_{35}
Contents: Curve 1: R_{35} in PB. Curve 2: About 3 hours after adding 0.1 ml. of GA (7.5 μM) to Curve 1. Curve 3: Difference spectrum, Curve 2—Curve 1. pH 7.0. At 30°.

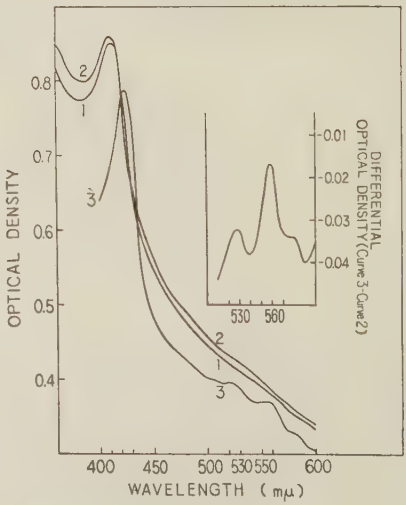


Fig. 6. Absorption spectra of R_{35}
Contents: Curve 1: R_{35} in PB. Curve 2: 80 minutes after adding 0.1 ml. of $M/100$ $K_3[Fe(CN)_6]$ to Curve 1. Curve 3: About 3 hours after adding 0.1 ml. of $Na_2S_2O_4$ (6 μM) to Curve 2. pH. 7.0.

From these results, it is conceivable that the dehydrogenation in R_{35} of

GA is connected to oxygen through some cytochrome system.

Above results also show that cytochrome(s) was present in DS_{35} almost in reduced form, but in R_{35} almost in oxidized form and these results seem to be in parallel with the observation that R_{35} had oxygen-consuming power for the substrate GA (completeness of the terminal electron transferring system), but DS_{35} almost lacked such activity.

b. Inhibition Studies—The oxygen consumption by R_{35} for GA was reduced to about 10 per cent of the control in the presence of 10^{-3} *M* NaCN and to zero at the concentration of 10^{-2} *M* of the inhibitor (Fig. 7).

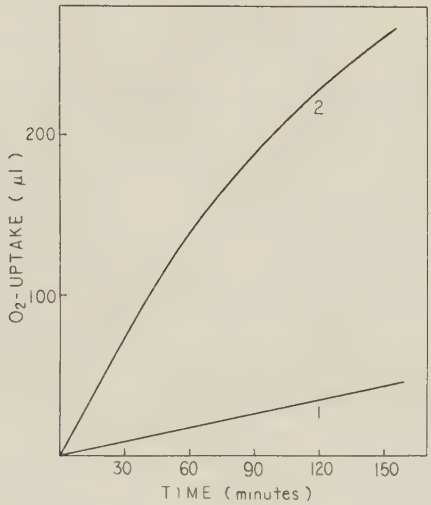


FIG. 7. Inhibitory effect of cyanide on the oxidation by R_{35} of GA

Contents: Curve 1: R_{35} 0.5 ml., GA 0.4 ml. (30 μM), NaCN 0.4 ml. (final conc. $10^{-3}M$), 10% KOH (NaCN-saturated) 0.2 ml. (center well), *M*/15 PB (PH 7.0) 2.5 ml. Curve 2: In the "contents" of Curve 1, *M*/15 PB in place of NaCN and ordinary), 10% KOH in place of NaCN-saturated KOH were used. Total 4.0 ml. At 30°.

However, in both concentrations of the inhibitor, R_{35} quickly decolorized CPIP as in the case without NaCN. This means that the dehydrogenation of GA was not affected by NaCN (Table III).

When air was introduced into the Thunberg tubes, the reduced CPIP was re-oxidized in the absence of NaCN and there was no re-coloration in the presence of NaCN where the CPIP oxidizing activity in R_{35} seemed to be inhibited.

From those results it is conceivable that the first step of GA oxidation by R_{35} was a dehydrogenation (by GA dehydrogenase) which was insensitive to NaCN and was connected to oxygen through some NaCN-sensitive

cytochrome system.

TABLE III

Effect of Cyanide on the Anaerobic Dehydrogenation by R_{35} of GA

	With NaCN		Without NaCN	
	Main	Control	Main	Control
R_{35}	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
GA (75 μM/ml.)	0.2	—	0.2	—
CPIP (1 μM/ml.)	0.2	0.2	0.2	0.2
NaCN ($10^{-5}M$/ml.) final conc. $10^{-3}M$	0.2	0.2	—	—
M/15 PB (pH 7.3)	0.9	1.1	1.1	1.3
Time required for decolorization at 30°.	3 min.	∞	3 min.	∞

DISCUSSION

Early in the studies (Report II of this series), it seemed propable that the oxidation of GA to GAA was analogous to the oxidation of glucose to gluconic acid by notatin, though this enzyme could not carry out the GA oxidation. In this paper, it was demonstrated that the activity of GA oxidation was localized in some particulate matter and that a dehydrogenase participated in the reaction through some cytochrome system.

When R_{35} was treated with digitonin solution the GA dehydrogenating activity was found in the supernatant fraction (DS_{35}), which, however, showed almost no oxygen consumption toward GA. Accordingly, in DS_{35}, the electron transferring chain from GA to oxygen seemed to be disconnected at some point(s). The color restoration of the reduced CPIP (non-autoxidizable) in the Thunberg test on the exposure to atmospheric oxygen (results, *4–b*) seemed to show the presence in R_{35} of so-called indophenol oxidase (cytochrome oxidase); in fact, R_{35} showed strong cytochrome oxidase activity by the "nadi reaction" and the indophenol color formed was also decolorized anaerobically by the addition of GA. So, it seemed to show the participation of cytochrome C-cytochrome oxidase in the GA oxidation system of R_{35} that the oxygen consumption toward GA and the color restoration of the reduced CPIP above mentioned were both inhibited by cyanide, whereas the dehydrogenation of GA itself was not affected by cyanide at all. The nature of GA dehydrogenase and the sequence of the electron transferring system connected with it are to be studied.

R_{35} oxidized both glucose and GA and it is so far not clear whether R_{35} contained two separate enzyme or only one enzyme of group specificity.

It may show the presence in this preparation of a small quantity of

GA that the oxygen consumption of about 0.08 atom per molecule of substrate CA was observed by R_{35}. (The presence of a small quantity of GA was observed also by the cation exchanger chromatography of S. Gardell (*21*).)

As CA itself seemed to be not oxidized and exert no effect on the oxidation by R_{35} of GA, it may be probable that GA alone can be determined enzymatically in the mixture of both aminosugars and both will be determined separately in combination with the Elson-Morgan method. The study on this problem is in progress.

SUMMARY

1. The glucosamine oxidizing activity in crude extract of *Pseudomonas fluorescens* was found to be localized in some particulate matter.

2. The particulates oxidized only glucosamine and glucose and had almost no activity toward several other sugars including galactosamine.

3. In these particulates, a dehydrogenase which might be called glucosamine dehydrogenase participated in the glucosamine oxidation through some cytochrome system.

4. The glucosamine dehydrogenating enzyme was extracted into high speed supernatants (about $100{,}000 \times g$) by digitonin treatment.

The author would like to express his thanks to Prof. Drs. S. Akabori, K. Okunuki of Osaka University and Y. Matsushima for their encouragement and also to Miss K. Yoshifuji for her assistance.

REFERENCES

(*1*) Leloir, L. F., and Cardini, C. E., *Biochim. et Biophys. Acta*, **20**, 33 (1956)
(*2*) Imanaga, Y., *J. Biochem.*, **44**, 69 (1957)
(*3*) Faulkner, P., and Quastel, J. H., *Nature*, **177**, 1216 (1956)
(*4*) Comb, D. C., and Roseman, S., *Biochim. et Biophys. Acta*, **21**, 193 (1956); Corab, P. G., and Roseman, S., *J. Biol. Chem.*, **232**, 807 (1958)
(*5*) Yamaha, T., and Asahina, M., *J. Japan. Biochem. Soc.*, **28**, 486 (1956)
(*6*) Wolfe, J. B., Morita, R. Y., and Nakada, H. I., *Arch. Biochem. Biophys.*, **64**, 480 (1956); Wolfe, J. B., and Nakada, H. I., *ibid.*, 489; Wolfe, J. B., Britton, B. B., and Nakada, H. I., *ibid.*, **66**, 333 (1957); Wolfe, J. B., Martinez, R. J., and Nakada, H. I., *ibid.*, **79**, 330 (1959)
(*7*) Maley, F., and Maley, G. F., *Biochim. et Biophys. Acta*, **31**, 577 (1959)
(*8*) Chou, J. C., and Soodak, M., *J. Biol. Chem.*, **196**, 105 (1952)
(*9*) Katz, J., Lieberman, I., and Barker, H. A., *J. Biol. Chem.*, **200**, 417 (1953)
(*10*) Leloir, L. F., and Cardini, C. E., *Biochim. et Biophys. Acta*, **12**, 15 (1953)
(*11*) Brown, D. H., *Biochim. et Biophys. Acta*, **16**, 429 (1955)
(*12*) Cardini, C. E., and Leloir, L. F., *J. Biol. Chem.*, **225**, 317 (1957)
(*13*) Comb, D. G., and Roseman, S., *Biochim. et Biophys. Acta*, **29**, 653 (1958)
(*14*) Glaser, L., *Biochim. et Biophys, Acta*, **31**, 575 (1959)
(*15*) Hochstein, L. I., Wolfe, J. B., and Nakada, H. I., *J. Am. Chem., Soc.*, **81**, 4111 (1959)

(*16*) Imanaga, Y., *J. Biochem.*, **44**, 819 (1957)
(*17*) Merrick, J., and Roseman, S., *Bact. Proc.*, p. 17 (1958)
(*18*) Imanaga, Y., *J. Biochem.*, **45**, 647 (1958)
(*19*) Meyer, K., and Smyth, E. M., *J. Biol. Chem.*, **119**, 507 (1937); Schubert, M., and Einbinder, J., *J. Biol. Chem.*, **185**, 725 (1950); Gardell, S., *Acta Chem. Scand.*, **5**, 195 (1951)
(*20*) Fischer, F. G., and Nobel, H. J., *Z. Physiol. Chem.*, **302**, 10 (1955)
(*21*) Gardell, S., *Acta Chem. Scand.*, **7**, 207 (1953)

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METABOLISM OF 4-METHYL-5-β-HYDROXY-ETHYLTHIAZOLE-S^{35} IN RATS*

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(Received for publication, February 8, 1960)

Several investigations have so far been reported on the metabolism of thiamine in rats (*1*, *2*) and rabbits, (*3*), but the metabolic fate of the thiazole moiety of thiamine, *i.e.* 4-methyl-5-β-hydroxyethylthiazole (Th)**, has not yet been published. The present authors studied the fate of Th-S^{35} in rats and the separation of the resulting urinary radioactive metabolites, and demonstrated that 4-methylthiazole-5-acetic acid (ThA) was the main urinary metabolite. The results are described below.

MATERIALS AND METHODS

Materials—Th-S^{35} was prepared by the method of Williams and Ronzio (*4*). The purity was cheched to be uniform radiochemically and spectroscopically by means of paper chromatography and paper eletrophoresis. The specific activity of the final product was 6.88×10^8 c.p.m. per mmol. About 200 g. Sprague-Dawley male rats maintained on a usual laboratory chow (Diet No. TH-1) were used.

Treatment of Rats—Th-S^{35} was dissolved in physiological saline so as to have an appropriate specific activity and the solution was adjusted to pH 7.0. After intraperitoneal or intravenous injection of Th-S^{35}, the rat was placed in an all-glass metabolism cage for the separate collection of urine, feces and expired air. Air was introduced into the cage at a rate of 500 ml. per minute and the issuing air was passed through traps containing 1 *N* NaOH and 0.1 *N* perchloric acid solutions. The rats were decapitated 5 minutes, 1 and 24 hours after injection.

Extracts of Tissues—After the collection of blood, various organs were excised, weighed and frozen in dry ice-acetone. These tissues were extracted twice with a cold perchloric acid solution according to the procedure of Hurlbert *et al.* (*5*).

Measurement of Radioactivity—An aliquot of urine or tissue extracts was directly plated on a planchet and dried, and the radioactivity was measured by a gas flow counter (Model D47 counter, Nuclear, Chicago). When the sample is anticipated to contain Th it must be acidified with perchloric acid solution beforehand, because Th is volatile in neutral or alkaline solution. The radioactive spots on the paper chromatogram were located by autoscanning with Actigraph II, Nuclear, Chicago.

Ion Exchange Chromatography—An Amberlite XE 64 (H form) column, 1×20 cm, was used for ion exchange chromatography. After 1 ml. of a sample was applied on the top

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* This report was presented at the 32nd General Meeting of The Japanese Biochemical Socienty (Osaka, 1–3, Nov., 1959)

** The following abbreviations are used. Th, 4-methyl-5-β-hydroxyethyl-thiazole; ThA, 4-methylthiazole-5-acetic acid; i.p., intraperitoneal; PcA, perchloric acid.

of the column, it was eluted first with water and then with 0.1 *M* perchloric acid solution at flow rate of about 0.5 ml. per min. When a large amount of the sample is applied, a larger column corresponding to the amount must be used. The linear gradient elution was carried out by the method of Lakshmanan and Lieberman (*6*).

Paper Chromatography— It was carried out by ascending method using filter peper "Toyo" No. 5 B and developing with the solvent systems shown in Table I. As shown

TABLE I

R_f Values of Th and ThA

Solvent System	Abbrev.	Th	ThA
n-Butanol-Acetic Acid-Water=12 : 3 : 5[a]	BuA	0.88	0.84
sec-Butanol saturated with Water	sec-Bu	0.93	0.34
n-Butanol-Pyridine-Water=1 : 1 : 1	BuPy	0.96	0.66
i-Propanol-Ammonia-Water=20 : 1 : 2	i-PrAm	0.90	0.38
Ethanol-Ammonia-Water=18 : 1 : 1	EtAm	0.85	0.44

Filter Paper, "Toyo" No. 5B., ascending. a) V/V.

by the *Rf* values of authentic sampless of Th and ThA (Table I), the BuA system proved unsuitable for the separation of the compounds. The radioactive spots on paper were located by radioactivity or ultraviolet absorption under irradiation with a 253 mμ lamp.

Paper Electrophoresis—The standard conditions were as follows. Filter paper "Toyo" No. 5B or No. 51, 8×45 cm, Solvent: 0.1 *N* acetic acid solution, 0.1 *M* acetate buffer (pH 4.6), or 0.1 *N* sodium acetate solution, Voltage gradient; 9 volt per cm., Time; 3 or 4 hours. The electrophoretic behaviours of Th and ThA are indicated in Table IV.

Microbiological Assays—Lactobacillus fermenti (ATCC 9338, IFO 3071) was used as the test microorganism for the assay of thiamine-replacing activity of ThA. The test medium was prepared according to the procedure of MaciasR (*7*). The bacterial growth was measured turbidimetrically after the incubation for 22 and 44 hours at 37°.

RESULTS

1. Distribution of Radioactivity after Injection of Th-S^{35}—Several experiments were conducted on the distribution of radioactivity in rats after injection of Th-S^{35} under various conditions (route, dose and time). The results all showed that the compound was rapidly absorbed and distributed throughout the body without any local concentration and then excreted largely in urine (Table II). In Experiment 1, 98 per cent of the recovered radioactivity was found in the urine collected during 24 hours, while less than 1 per cent was observed in total viscera, skeletal muscles and expired air. The authors then intended to clarify the chemical natures of the urinary metabolites.

2. Separation of Urinary Metabolites—Preliminary experiments showed that the metabolic pattern of urinary metabolites of rats given a large dose of the compound was the same as that of rats administered with a small dose. Therefore, the urines from six rats administered each with 100 mg.

TABLE II

Per Cent Recovery of the Radioctivity in Rats after Injection of Th-S^{35}

Exp. No.	1	2	3	5
Rat (No., Sex, Weight)	1, ♂, 205 g.	6, ♂, 165 g.	1, ♂, 225 g.	6, ♂, 190 g.
Injection Route	i.p.	i.p.	i.p.	i.p.
Dose per Rat, mg.	0.94	100	100	100
c.p.m.	2.29×10^6	4.11×10^6	4.19×10^6	6.93×10^6
Hours after Injection	24	24	1	24
Per Cent Recovery				
Organs[1]	0.34%	—	5.6%	—
Skeletal Muscle	0.0	—	—	—
Blood[2]	0.15	—	4.4	—
Expired Gas	1.2	—	—	—
Urine	84.9	97.6	24.3	100.1
Total	86.6	97.6	34.3	100.1

1) Organs tested; Brains, lung, heart, liver, spleen, kidney and small intestine.

2) Calculated on the basis of the assumption that the total blood volume is 8 per cent of body weight.

of the compound (4.1×10^6 c.p.m.) (Expt. 2) were pooled after 24 hours and concentrated to about 10 ml. (1/10 volume) under reduced pressure. In order to remove inorganic salts, the concentrate was subjected to solvent extractions. Although Th is readily extractable with chloroform, the radioactivite metabolites were hardly extracted with the solvent, but they were extracted with phenol or acidified *n*-butanol almost quantitatively (Table III). This property suggests that the majority of the urinary radioactive substances are different from Th.

TABLE III

Solvent Extraction of S^{35}-active Substances from the Urine.

Solvent	Per Cent Partition[1]	
	Solvent Layer	Aqueous Layer
Chloroform, pH 6.0	1.8%	98.2%
Chloroform, pH 9.0	0.5	99.5
Phenol, pH 7.0	99.0	1.0
n-Butanol, pH 2	91.5	8.5

1) Extracted thrice.

Then, a *n*-butanol extract, after removal of the solvent under reduced

pressure, was chromatographed on an Amberlite, XE 64 column. Fig. 1 shows that more than 95 per cent of the radioactivity of the substance applied is found in the C-1 fraction eluted with water. In the preliminary experiments, Th was found to be adsorbed on the column and not to be eluted with water, unless the eluent was acidified. Here again, the compound in the C-1 fraction was found not to be identical with Th. This was further confirmed by the fact that the R_f-value and eletrophoretic mobility of the compound are different from those of Th (See Section 3).

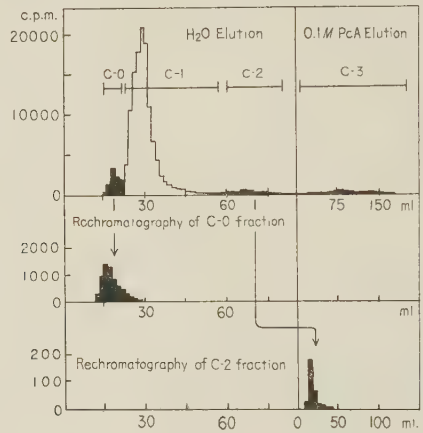

Fig. 1. Ion exchange chromatographic separations of the urinary metabolites.
The Sample: *n*-butanol extract of the urine obtained in Expt. 5.
The Conditions for chromatography: See Text.

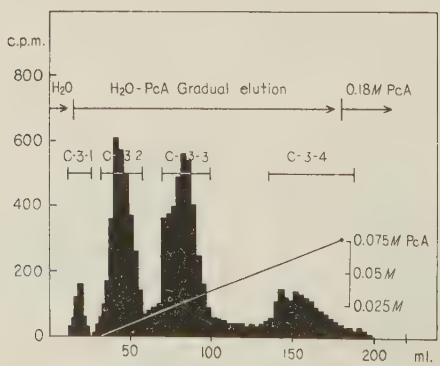

Fig. 2. Rechromatography of the C-3 fraction.
The conditions for chromatography: See text.

From the concentrate of the pooled fraction of C-1 radioactive needles were obtained. This compound was tentatively named substance A, whose

specific activity was 3.6×10^4 c.p.m. per mg. and did not change during repeated recrystallizations. Later, the compound was identified as 4-methylthiazole-5-acetic acid. The detailed properties of the compound and its identification will be described in Section 3.

In the ion exchange chromatographic separation, there were found three minute radioactive peaks, namely, C-0, C-2 and C-3, beside the main peak, C-1. The C-0 fractions were pooled and rechromatographed under the same conditions as before to give a uniform peak. In the rechromatography of the C-2 fraction, the radioactive substance was adsorbed on the column to be eluted with 0.1 *N* perchloric acid (Fig. 1). The C-3 fraction was also rechromatographed by means of a gradient elution technique and separated into four radioactive peaks, *i. e.* C-3-1, C-3-2, C-3-3 and C-3-4 fractions (Fig. 2). These six fractions contained only less than 1 per cent of the radioactivity of the substance applied, respectively. So it seemed difficult to isolate these active compounds in crystalline form. Therefore, they were subjected to paper chromatographic and electrophoretic identifications. The results are summarized in Table IV. The C-0 fraction

TABLE IV

R_f Values and Electrophoretic Behaviors of S^{35}-active Components in the Fractions Obtained by Ion Exchange Chromatography of the Urine.

Fraction	R_f Values	Electrophoretic Behavior[1]		
		pH 2.3	pH 4.6	pH 7.9
C-0	0.02, 0.26, 0.56, 0.77	-0.4, +1.3	+3.8	-0.9, +3.9
C-1	0.38 (ThA)	-6.0	+3.8 (ThA)	+4.5
C-2	0.14, 0.90	—	-0.5	-0.3, +2.8
C-3	0.39, 0.78, 0.84, 0.90	—	-4.5, -2.8, -0.5 +3.9	—
C-3-1	0.01, 0.35 (ThA)	—	-0.2, +4.0 (ThA)	—
C-3-2	0.18	-4.0	+3.8	—
C-3-3	0.02, 0.44, 0.73	—	-5.3, -4.0, -1.4	-0.3
C-3-4	0.36 (ThA), 0.96 (Th)	—	-0.7 (Th), +3.8 (ThA)	—
Th	0.90—0.93	-13.0	-0.8 — -1.0	-0.3
ThA	0.35—0.37	- 6.0	+3.8 — +4.0	+4.5

1) Migration distances, cm., from the origin and designated+sign when migrated toward the anode and *vice versa*. 300 volt/40 cm., 3 hours in 0.1 *M* acetic acid, 0.1 *M* acetate buffer, pH 4.6 or 0.1 *M* sodium acetate. Underline denotes the predominant component.

contained at least two radioactive compounds more acidic than ThA. The main component of the C-2 fraction, though not yet identified, is more acidic than Th but more basic than ThA and its R_f was 0.14 in i-PrAm system. The C-2 fraction also contained a small amount of Th as an additional minor component. The main component of C-3-1 fraction was

identified as ThA, but the amount was only less than 0.05 per cent of ThA found in the main fraction, C-1. The active compound contained in C-3-2 fraction showed similar electrophoretic mobility to that of ThA but its R_f-value was widely different from that of ThA. In the C-3-3 fraction there were found three unidentified components, of which two were more basic than Th. These results led us to the conclusion that of the radioactive urinary excreta over 95 per cent was recovered as ThA and 1 per cent or less as intact Th. Besides these two compounds, at least six unidentified metabolites including acidic and basic substances were found in much smaller amounts, but the result is not still conclusive.

3. Properties and Identification of Substance A—As reported in Section 2, colorless needles were obtained from the C-1 fraction. The compound, named, tentatively "substance A", was recrystallized from boiling water without any decrease in the specific activity. One hundred and twenty-two mg. crystallines of the substance were obtained from the urine of Experiment 2 (Table I). The properties of the substance are described below.

1) Melting Point: 191°.
2) Elementary Analysis (Expected formula, $C_6H_7O_2NS$)—

	C	H	N	S
Calcd.:	45.84	4.49	8.91	20.40
Found:	45.40	4.79	8.80	20.19

3) Molecular Weight by Burger Method: 145±15 (Expected, 157
4) Ultraviolet Absorption Spectrum

$$\lambda_{max}^{0.1\,N\ HCl}\ 256\ m\mu\ (4570);\quad \lambda_{max}^{0.1\,N\ NaOH}\ 252\ m\mu\ (4500).$$

These figures are closely similar to those of Th (Fig. 3). Therefore, substance A was strongly expected to contain thiazole ring.

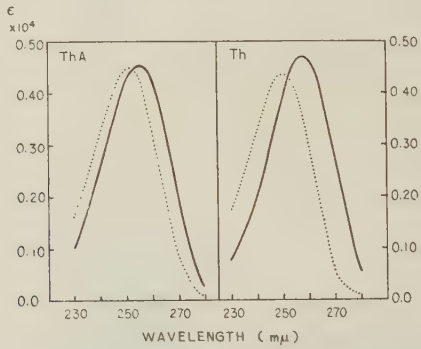

FIG. 3. Ultraviolet absorption spectra of Th and ThA.
—— 0.1 *N* HCl, ------ 0.1 *N* NaOH

5) Infrared Absorption Spectrum—The infrared absorption spectrum of substance A (Fig. 4) suggests the absence of the hydroxyl group.
6) Papar Chromatographic Behaviors—Th gave almost the same *Rf*-value both in butanol-acetic acid and in butanol-pyridine solvent systems. On the other hand, the value of ThA in a solvent containing such a base as pyridine or ammonia is lower than in butanol-acetic acid system (Table I, Fig. 5A), where the values

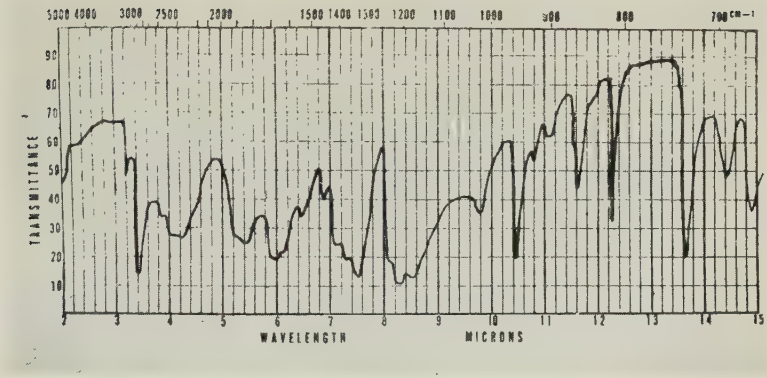

FIG. 4. Infrared absorption spectrum of Substance A.

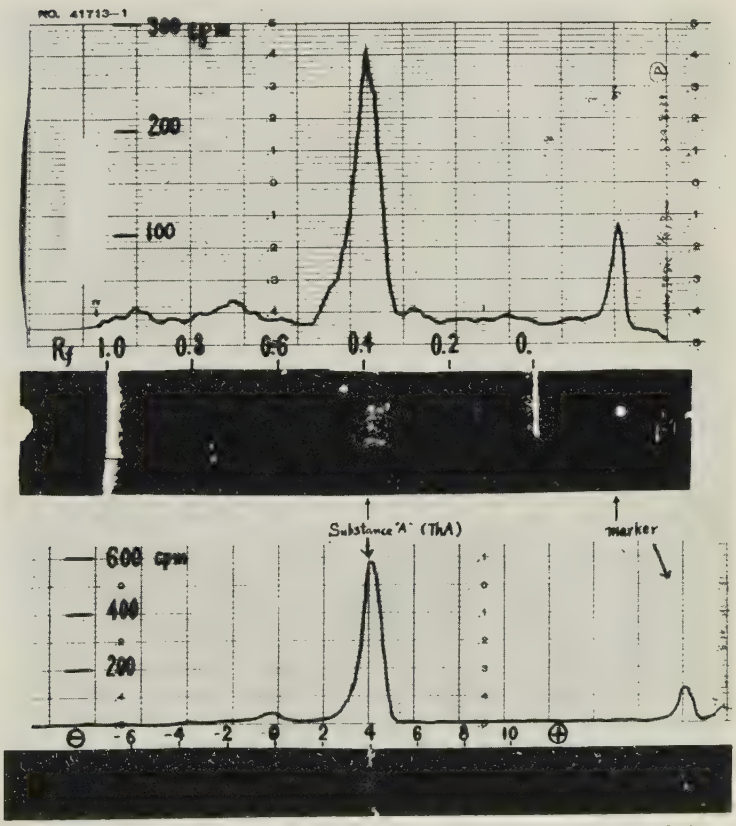

FIG. 5A and 5B. Paper chromatography and paper electrophoresis of the urine obtained in Exp. 5 (Table II).

A i-PrAm system. (See the Text of Table I)

B 0.1 *M* Acetate buffer, pH 4.6; 9 volt/cm., 4 hr. (See the Text of Table IV)

Photographs show the autoscanning of the radioactivity and ultraviolet absorption spots.

of both Th and ThA are quite identical. This *Rf* shift of substance A in acidic and alkaline solvents suggests the presence of a weak acidic group in the compound.

7) Paper Electrophoresis—Paper electrophoretic behaviors of Th and substance A are shown in Table IV and Fig. 5B. Th migrates only toward the cathode in acidic medium, while substance A migrates to the cathode at pH 2.9 and to the anode at pH 4.6 or 7.9. This also attests the presence of a weak acidic group.

From all these results substance A was assumed to be an oxidation product of Th, namely 4-methylthiazole-5-acetic acid ($C_6H_7O_2NS$). The value of the elementary analysis and the molecular weight well agree with this empirical formula. The melting point is also close to that (189°) reported by Cerecedo *et al.* (*8*). Later the authors could obtain an authentic sample of ThA, which was synthesized by Dr. Hirano and Mr. Tsujikawa according to the method of Cerecedo *et al.* (*8*). Substance A showed no depression in m.p. when admixed with the authentic sample, and its ultraviolet and infrared absorption spectra, R_f-value and electrophoretic mobility were all well coincident with those of the synthetic ThA. The authors, therefore, conclusively confirmed that substance A was identical with ThA.

By the microbiological assays using *Lactobacillus ferment* which requires thiamine (1–50 mμg. per ml.) or Th (20–200 μg. per ml.) for the growth, ThA was found to be quite inactive in the concentration of less than 1 mg. per ml, regardless to the supplement of 2-methyl-4-amino-5-hydroxymethylpyrimidine.

DISCUSSION

Of Th-S^{35} injected, more than 24 per cent of the radioactivity was excreted in urine in one hour, and 85–95 per cent within 24 hours without any accumulation in particular organ tissues. In addition, ThA accounted for about 95 per cent of the radiosulfur in urine. From these facts it is apparent that the oxidation of Th to ThA and excretion of the latter take place rather rapidly. In the authors' experiments, it was not undertaken to obtain accurate balances of the radioactivity administered and recovered, but the lower recoveries in the tests of short periods may have resulted from self-absorption due to direct measurement, omission of the measurement of the perchloric acid insoluble fractions, incompleteness of the extraction, *etc.*

The present experimental data reveal that ThA is the main metabolite of Th in rats. The occurrence of the compound in natural products has never been reported, but it is well known that the biological oxidation of a primary alcohol to the corresponding carboxylic compound is one of the most wide-spread detoxication mechanisms. Kofler and Sternbach (*9*), Kraut and Wildeman (*10*) and Iacono and Johnson (*2*) reported that thiamine administered to mammals was excreted in part as thiamine carboxylic acid, and this biological conversion may be due to the same mechanism as above. A similar metabolism was also reported by

Shintani (*11*) on 2-methyl-4-amino-5-hydroxymethylpyrimidine, the pyrimidine moiety of thiamine, which was excreted as the corresponding carboxylic acid when injected in rabbits.

Although Verett *et al.* (*3*) and Iacono *et al.* (*2*) announced the urinary excretion of Th following the administration of S^{35}- or C^{14}-labeled thiamine, they did not obtain any experimental evidence suggesting the occurrence of ThA. Judging from the authors' results indicating the easy conversion of Th to ThA in rats, the possibility would be anticipated that a part of thiamine administered might be excreted in urine after conversion to ThA *via* Th. The experimental procedures used by Verett *et al.* (*3*) is unsuitable for the separation and detection of ThA. On the other hand, the fluorescent spot No. 15 reported by Iacono *et al.* (*2*) has similar R_f values in BuA and *sec*-butanol systems to those of ThA found by the authors, but it is not justifiable to infer that spot No. 15 corresponds to ThA, for the latter does not emit fluorescence.

SUMMARY

1. In the present study on the fate of S^{35}-labeled 4-methyl-5-β-hydroxyethylthiazole (Th) in rats, 85–95 per cent of the radioactivity administered intraperitoneally or intravenously was excreted into urine within 24 hours and there was found no accumulation of the radioactivity at any organ investigated.

2. The radioactive urinary metabolites were investigated by ion exchange chromatography, paper chromatography and paper electrophoresis. Urinary excretion pattern of Th-S^{35} in the case of a small dose (1 mg. per rat) was the same as in a large dose (100 mg. per rat). The majority (about 95 per cent) of the metabolites was isolated, characterized and identified with 4-methylthiazole-5-acetic acid. Several radioactive components including unchanged Th were also demonstrated, although each amounts to only one per cent or less.

The authors are deeply iedebted to Mr. T. Iida for his technical assistance, to Dr. Kanzawa and Mr. Nishikawa for the infrared spectroscopy and to Dr. Tanabe, Mrs. Kan, Kashiwagi, Suzuki and Tsukamoto for elementary analysis and to Dr. Hirano and Mr. Tsujikawa for the synthesis of ThA.

REFERENCES

(*1*) Macarthy, P. T., Cerecedo, L. R., and Brown, E. V., *J. Biol. Chem.*, **209**, 611 (1954)
(*2*) Iacono, J. M., and Johnson, B. C., *J. Am. Chem. Soc.*, **79**, 6321 (1957)
(*3*) Verrett, M. J., and Cerecedo, L. R., *Proc. Soc. Exptl. Biol. Med.*, **98**, 509 (1958)
(*4*) Williams, D. L., and Ronzio, A. R., *J. Am. Chem. Soc.*, **47**, 2409 (1952)
(*5*) Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954)
(*6*) Lakshmanan, I. K., and Lieberman, S., *Arch. Biochem. Biophys.*, **53**, 258 (1954)

(*7*) MaciasR, F. M., *Appl. Microbiol.*, **5**, 249 (1957)
(*8*) Cerecedo, L. R., and Tolpin, J. G., *J. Am. Chem. Soc.*, **59**, 1660 (1937)
(*9*) Kofler, M., and Sternbach, L., *Helv. Chem. Acta.*, **24**, 1014 (1941)
(*10*) Kraut, H., and Wildeman, L., *Biochem. Z.*, **321**, 368 (1951)
(*11*) Shintani, S., *J. Pharmac. Soc. Japan*, **77**, 781 (1957)

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STUDIES ON THE STRUCTURE AND ENZYMATIC FUNCTION OF LYSOZYME

I. ENZYMATIC ACTION OF LYSOZYME ON GLYCOL CHITIN

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Lysozyme is a most promising protein for studies of physical and chemical structure, because of its ready availability, its simple structure and low molecular weight. However, the enzymatic properties of lysozyme are not well defined due to the very complex nature of the substrate, *i.e.*, the cell-wall substance of bacteria. Therefore, if a substrate having a simple chemical constitution were available, lysozyme would become a much more favorable protein for studies of physical and chemical structure in relation to enzymatic activity.

We reported in the previous communication (*1*) that glycol chitin can be used as a substrate of lysozyme and that the activity of lysozyme can be readily determined by the measurement of the viscosity change of glycol chitin after addition of the enzyme. This paper is mainly concerned with the general properties of the enzymatic action of lysozyme on glycol chitin.

EXPERIMENTAL

Materials—Lysozyme was prepared from hens' egg white by direct crystallization. It was recrystallized four times and finally lyophilized. Glycol chitin was kindly supplied by Dr. Senzyu, who prepared it by glycolation of chitin with ethylene oxide (*2*). Glycol chitosan was also supplied by Dr. Senzyu.

Methods—Viscosity was measured with an Ostwald viscosimeter. Colorimetric determinations of reducing power and N-acetylglucosamine were performed by the methods of Somogyi (*3*) and Elson-Morgan (*4*), respectively. For both methods a solution of glucosamine hydrochloride was used as a reference. The concentration of lysozyme was determined by measuring the increase in refractive index using a Brice-Phoenix's differential refractometer ($dn/dc=0.195$ at 436 mμ) (*5*).

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RESULTS AND DISCUSSION

Viscosity Change and Development of Reducing Power and the Elson-Morgan Reaction—The action of lysozyme on glycol chitin in $M/15$ phosphate buffer (pH 5.5) at 33° was investigated by measuring the intensity of the Elson-Morgan reaction, the increase in reducing power, and viscosity change. The results obtained are shown in Fig. 1. The reducing power and the intensity of color produced by the Elson-Morgan reaction are given as values compared with reference glucosamine hydrochloride.

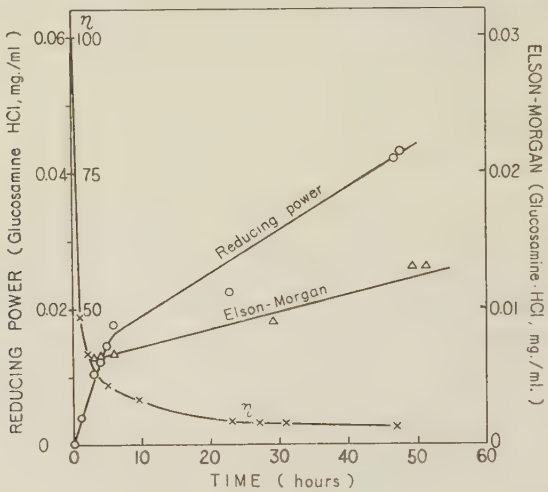

FIG. 1. Change with time of viscosity, reducing power, and intensity of the Elson-Morgan reaction of glycol chitin under the action of lysozyme. Concentration of glycol chitin: 0.26 per cent; concentration of lysozyme: 24 μM (assuming that the molecular weight of lysozyme is 14,500). pH 5.5 ($M/15$ phosphate buffer), 33°.

It is shown in Fig. 1 that the increase in reducing power and in the intensity of color produced by the Elson-Morgan reaction was concomitant with the viscosity decrease. Recently, Berger and Weiser (*6*) reported that lysozyme has β-glucosaminidase activity because of its ability to hydrolyze chitin. The results presented here confirm their results.

It is uncertain whether glycol N-acetylglucosamine is liberated, because the Elson-Morgan reaction is in no way specific for this monomer. The rapid decrease in viscosity of glycol chitin solution after addition of lysozyme suggests that lysozyme may split glycol chitin molecules at random. The increase in reducing power after 52 hours is equivalent to 0.05 mg./ml. of glucosamine hydrochloride (Fig. 1). This figure corresponds to only 2 per cent of the initial concentration of glycol chitin used (2.6 mg./ml.), assuming that glycol chitin is completely hydrolyzed to N-acetylglucosamine

by lysozyme. Recent investigations on the hydrolyzates of bacterial cell-wall substances under the action of lysozyme show that there is no N-acetylglucosamine but higher molecular weight fragments attached by N-acetylaminosugar-reacting groups (*7*, *8*).

Of the changes during the hydrolysis of glycol chitin by lysozyme, the viscosity change was the most marked. Therefore, the viscosimetric method was employed for the determination of lysozyme activity throughout the following experiments.

Optimum pH, Optimum Temperature, and the Effect of pH on the Stability of Lysozyme—The pH-activity curve which was determined by the viscosimetric method (see ref. (*1*)) is shown in Fig. 2. The ordinate in this figure represents the initial velocity (v_0) of the decrease in viscosity of glycol chitin. It can be seen that the optimum for lysozyme activity lies at pH 3.5. The value for the optimum pH as determined by lysis of bacteria was very different, being between pH 5 and 7.4.

The optimum temperature, as determined by using glycol chitin is 50° at pH 3.5, whereas that determined by lysis of bacteria is between 55° and

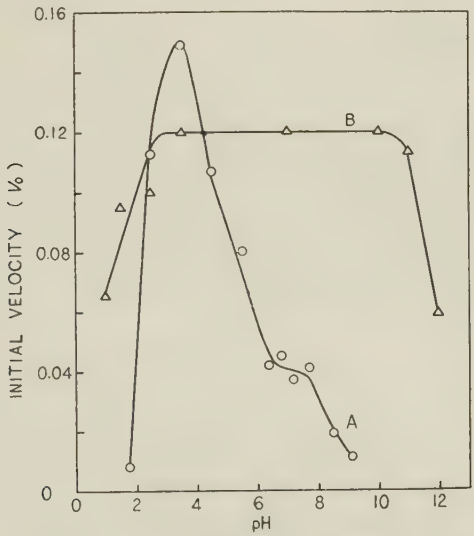

Fig. 2. Curve A: the activity was tested at the pH values shown. pH 4.5–9.1: *M*/15 phosphate buffer, pH 1.75–3.5: *M*/15 glycine buffer. Curve B: the enzyme was exposed to the pH values shown for 1 hour at 33° and the activity was then tested at pH 3.5 at 33°.

60° (*9*). It is interesting to note that the optimum temperature corresponds to the temperature above which the foaminess of lysozyme solution increases (*10*, *11*).

As pointed out in the previous communication (*1*,), it is quite interesting that the enzymatic properties of a liquifying chitinase obtained from

Aspergillus niger by Ohdakara (*12*), with glycol chitin as substrate, resembles that of lysozyme.

To examine the effect of pH on the stability of lysozyme, the activity of lysozyme was determined at pH 3.5 after preincubation at various pH's (as 0.18 per cent solution) at 33° for 1 hour. Activity was measured with 0.042 per cent of glycol chitin and final concentration of lysozyme of 0,4 μM. The results are shown in Fig. 2. It is obvious that lysozyme is unstable below pH 2.5 and above 10.0.

Effect of Sodium Chloride and Various Buffers—The effect of the concentration of sodium chloride on the activity of lysozyme is shown in Fig. 3. The activity increased about 10 per cent in the presence of 0.1 and 1.0 M sodium chloride. However there was no effect with change in the composition of the buffer (M/10 phosphate, citrate, acetate) at pH 5.6.

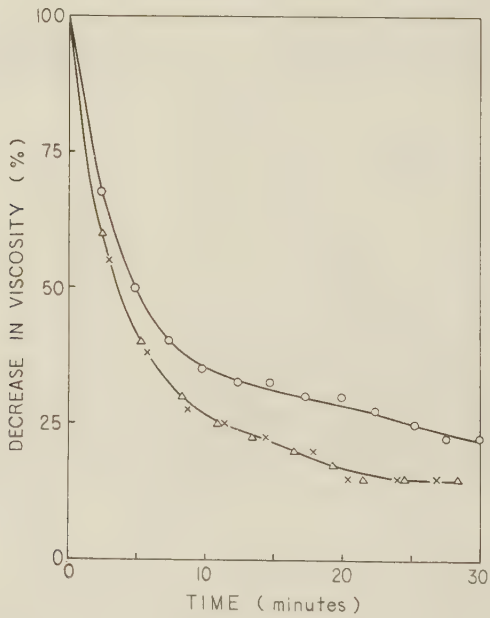

Fig. 3. The effect of the concentration of sodium chloride on the activity of lysozyme. Concentration of glycol chitin: 0.031 per cent, final concentration of lysozyme: 3.8 μM. pH 7.3 (M/15 phosphate buffer), 33°, ○, without NaCl; △, 0.1 M NaCl; ×, 1.0 M NaCl.

Inactivation of Lysozyme by Urea—Inactivation of lysozyme by urea has been studied by Léonis (*13*) and by Dickman ald Procter (*14*) using lysis of bacteria as a measure of activity. However since the lytic process is enhanced by urea, we examined the inactivation of lysozyme by urea using glycol chitin as substrate.

After lysozyme (0.14 per cent) had been preincubated with various

concentrations of urea (pH 6.4, $M/10$ phosphate buffer) at 33°, the activity of lysozyme was determined in the presence and absence of urea, using glycol chitin as a substrate. The results are shown in Fig. 4.

When measured in urea solutions the activity gradually decreased with increase in urea concentration (Curve I). This curve, however, does not demonstrate inactivation of lysozyme itself by urea, because an interaction between the substrate and urea must also be taken into account. The ac-

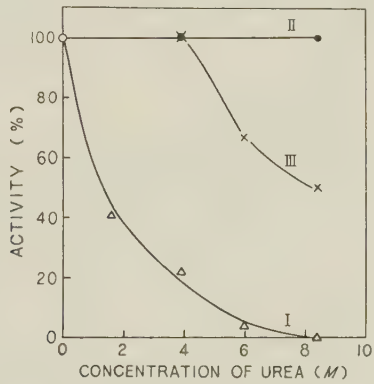

Fig. 4. Effect of urea on activity of lysozyme. Curve (I): Activity measured in urea solutions after lysozyme had been preincubated with urea for 1 hour. Curve (II): Activity measured in the absence of urea after lysozyme had been preincubated with urea solutions for 4 hours. Curve (III): The same as curve (II) except that the preincubation period was 25 hours. Activity is expressed as per cent of the initial velocity of native lysozyme.

tivity determined in the absence of urea after lysozyme had been preincubated with urea solutions for 4 hours was the same as that of the native enzyme. The activity determined in the absence of urea after 25 hours incubation (Curve III) decreased at concentrations of urea higher than 4 M. However after preincubation in 6 or 8 M urea for 25 hours, the lysozyme activity on glycol chitin was depressed in the initial stage of the hydrolysis and gradually recovered thereafter.

The relationship between the inactivation and denaturation of lysozyme by urea will be described in detail in the next paper.

Effect of Heating on Lysozyme Activity—As described above, the optimum temperature of lysozyme is 50°. On the contrary, we found that pretreatment of lysozyme by heat caused considerable activation. For instance, after lysozyme had been preincubated at 50° for 4 hours the activity measured at 33° is almost twice as great as that of the intact enzyme (Fig. 5). The optimal period of heat treatment required for maximal activation of lysozyme varied with the temperature used. The optimal period shortened with rise in temperature. Pretreatment of lysozyme by heat did not cause

inactivation at temperatures below 80°. In boiling water, however, lysozyme was completely inactivated after 75 minutes. Details of the mechanism of heat activation of lysozyme will be described in the near future.

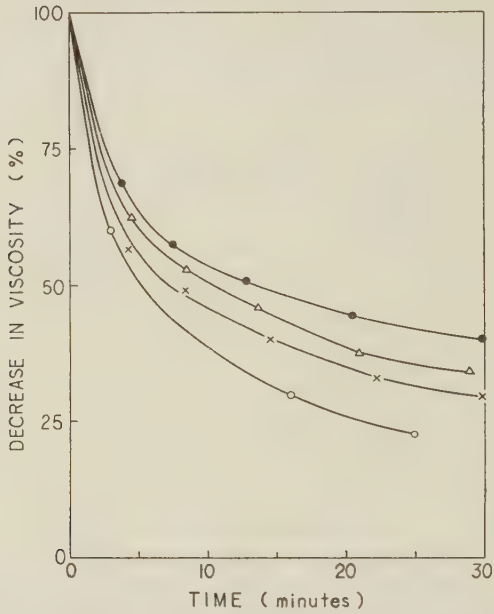

FIG. 5. Effect of heat treatment on lysozyme activity. Lysozyme (0.57 per cent) in $M/15$ phosphate buffer (pH 5.5) was incubated for 0 (●), 1 (△), 2.5 (×) and 4 (○) hours at 50° and then the activity was determined using glycol chitin as a substrate (0.04 per cent) (pH 5.5) at 33°. Final concentration of lysozyme: 0.76 μM.

Action of Lysozyme on Glycol Chitosan—Berger and Weiser (*6*) reported that lysozyme can not hydrolyze chitosan. We also found that glycol chitosan is not hydrolyzed by lysozyme. Therefore, lysozyme seems only to act on a polymer of an N-acetyl derivative of glucosamine and not on the deacetylated polymer.

SUMMARY

Using glycol chitin as substrate, several enzymatic properties of egg white lysozyme were examined. The results were as follows.

1. A rapid decrease in viscosity and increase in reducing power and intensity of color produced by the Elson-Morgan reaction were observed on hydrolysis of glycol chitin by lysozyme.

2. The optimum pH and temperature for lysozyme activity were found to be at pH 3.5 and 50°, respectively. Lysozyme was unstable at pH's below 2.5 and above 10.0.

3. The stability of lysozyme decreased in solution of urea more concentrated than 4 *M*.

4. Pretreatment of lysozyme by heat increased its activity. Heat inactivation was not observed at temperature below 80°. However, in boiling water lysozyme was inactivated.

5. Glycol chitosan was not hydrolyzed by lysozyme.

The authors wish to express their hearty thanks to Prof. T. Isemura for his kinds support, and to Dr. R. Senzyu of Kyushu University who supplied glycol chitin and glycol chitosan, and to Dr. Y. Imanaga of Nara Women's University who supplied glucosamine hydrochloride.

REFERENCES

(*1*) Hamaguchi, K., and Funatsu, M., *J. Biochem.*, **46**, 1659 (1959)
(*2*) Senzyu, R., and Okimasu, S., *J. Japan. Agr. Chem. Soc.*, **23**, 432 (1950)
(*3*) Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945); 195, 19 (1952)
(*4*) Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933)
(*5*) Halwer, M., Nutting, G. C., and Brice, B. A., *J. Am. Chem. Soc.*, **73**, 2786 (1951)
(*6*) Berger, L. R., and Weiser, R. S., *Biochim. et Biophys. Acta*, **26**, 517 (1957)
(*7*) Schütte, E., and Krisch, K., *Z. physiol. Chem.*, **311**, 121 (1958)
(*8*) Salton, M. R. J., *Biochim. et Biophys. Acta*, **22**, 495 (1956)
(*9*) Fromageot, C., *Bull. soc. chim. biol. Supple.*, **11–12**, 63 (1948)
(*10*) Hamaguchi, K., *J. Biochem.*, **44**, 695 (1957)
(*11*) Hamaguchi, K., *Bull. Chem. Soc. Japan*, **31**, 123 (1958)
(*12*) Ohdakara, A., "The Meeting of the Agricultural Chemical Society of Japan", Tokyo, April, 1959 and personal communication
(*13*) Léonis, J., *Arch. Biochem. Biophys.*, **65**, 182 (1956)
(*14*) Dickman, S. R., and Procter, C. M., *Arch. Biochem. Biophys.*, **40**, 364 (1952)

The Journal of Biochemistry, Vol. 48, No. 3, 1960

STUDIES ON THE STRUCTURE AND ENZYMATIC FUNCTION OF LYSOZYME

II. EFFECT OF UREA ON LYSOZYME

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The effect of urea on some enzyme proteins has been studied to elucidate the activity of the enzymes in relation to their secondary and tertiary structure (*1*). However, such investigations have not progressed with lysozyme because the lysis of bacteria, by which the activity of this enzyme has been determined, is enhanced by urea. Thus, studies on the effect of urea on the activity of lysozyme reported by Léonis (*2*) and Dickman and Procter (*3*) were only preliminary. In the previous paper (*4*), we reported the effect of urea on the activity of lysozyme, using glycol chitin as substrate. The denaturation of lysozyme by urea has been investigated by Léonis (*2*), Glazer (*5*), and one of the present authors (*6*, *7*, *8*). In addition to the experiments published previously, we have examined the intrinsic viscosity and optical rotatory dispersion of lysozyme in urea solutions. In this paper, the relationships between the changes in activity and the physicochemical properties of lysozyme in urea solutions are summarized.

EXPERIMENTAL

Materials—Lysozyme was prepared from hens' egg white by direct crystallization. It was recrystallized three times and finally lyophilized. Reagent-grade urea was used without recrystallization.

Methods—Viscosity was measured with an Ostwald viscosimeter. Optical rotatory dispersion was measured at 33° between 420 and 600 mμ in increments of 20 mμ with a Hitachi photoelectric spectropolarimeter. The temperature was controlled by circulating water from a constant temperature bath through a jacketed polarimeter tube. The concentration of lysozyme used for the measurement of the dispersion was 1.46 per cent. The concentration of lysozyme was determined by the micro-Kjeldahl method or by measuring the refractive index increment using a Brice-Phoenix differential refractometer (0.195 at 436 mμ) (*9*). Activity of lysozyme was measured using glycol chitin as substrate (*4*).

RESULTS

The intrinsic viscosity and optical rotatory dispersion were measured

at pH 6.4 ($M/30$ phosphate buffer) and 33°. When the specific viscosities are plotted against the concentration of lysozyme, a straght line is obtoined which passes through the origin. This line is independent of the concentration of urea, as shown in Fig. 1. The intrinsic viscosity of the lysozyme

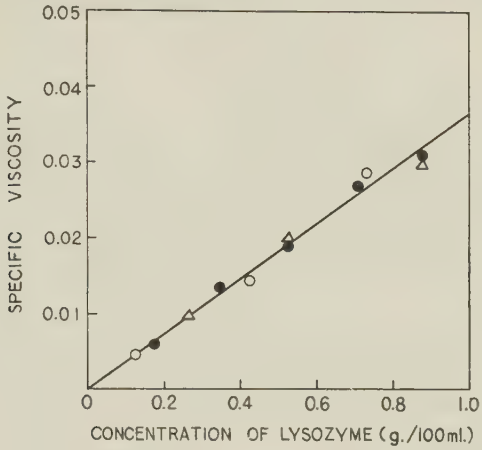

Fig. 1. Plot of specific viscosity against concentration of lysozyme in 0 (○), 4 (△), and 8 M urea (●). pH 6.4 ($M/30$ phosphate buffer). 33.0°.

Table I

The Intrinsic Viscosities, Dispersion Constants, and Activities of Lysozyme at varying Urea Concentrations

(33°, pH 6.4)

Concentration of urea (M)	Intrinsic viscosity $[\eta]$, (g./100 ml.)$^{-1}$		Dispersion constant, λ_c (mμ)		Activity[1] (per cent)	
	Incubation time (hours)					
	1	26	1	26	4	25
0	0.037		261		100	100
4	0.036				100	100
6			260	260	100	68
8	0.037	0.037	255	255	100	52[2]

1) Ref. (*4*). Activity measured in the absence of urea after lysozyme had been preincubated with urea solutions for 4 and 25 hours.

2) The activity was not recovered by dilution to 0.8 M urea after lysozyme was incubated in 8 M urea for 25 hours.

was not changed after incubation in 4 or 8 M urea at 33° for 26 hours.

The optical rotatory dispersion of lysozyme in 0, 6 and 8 M urea obeys a simple Drude law. The dispersion constant, λ_c, was obtained by the least square method from the Lowry plots ($1/[\alpha]$ vs. λ^2.) The results

obtained are shown in Table I. The dispersion constant was not changed after lysozyme had been incubated for 26 hours at 33°. The dispersion constant was only slightly decreased by increase in urea concentration.

The effect of urea on the intrinsic viscosity, dispersion constant, and activity of lysozyme are summarized in Table I.

DISCUSSION

The intrinsic or reduced viscosity of native lysozyme was determined by Glazer (*6*), Léonis (*2*), Yang and Foster (*10*), Jirgensons (*11*), and one of the present authors (*8*). These values obtained were very divergent, being between 0.025 (*2*) and 0.05 (*10*). Previously however, one of the authors found that the reduced viscosity was 0.035 (*8*), which is in good agreement with the value for the intrinsic viscosity obtained in the present experiment.

The optical rotatory dispersion constant of native lysozyme was reported to be 257 (*12*) or 254 mμ (*13*). These values are in good agreement with the present value of 260 mμ.

Fig. 2 summarizes our results on the denaturation and inactivation of lysozyme by urea.

As shown in Table I and Fig. 2, the intrinsic viscosity in 8 *M* urea (pH 6.4) at 33.0° was the same as that of native lysozyme. Furthermore, compared with other proteins in urea solutions, the decrease in optical rotatory dispersion constant of lysozyme in 8 *M* urea was very small. Therefore the secondary and tertiary structures of the lysozyme molecules are not destroyed in 8 *M* urea. The decrease in the dispersion constant in 8 *M* urea was so small that lysozyme cannot be considered to be denatured. Nevertheless, the stability of lysozyme activity decreased in solutions of urea more concentrated than 4 *M* at 33°.

Previously, one of the present authors found that the reduced viscosity increased with rise in temperature in over 4 *M* urea (*7*) and that the area of the lysozyme monolayer spread from the urea solutions was also increased in over 4 *M* urea (*6*, *8*). Therefore, though the inactivation of lysozyme (curve (III)) does not correlate with the intrinsic viscosity (curve (IV)) and dispersion constants (curve V)) at the same temperature, the inactivation is parallel to the change in reduced viscosity at higher temperatures (curve (VII) and (VIII)) and to the expansion of the monolayer (curve (IX)).

Therefore lysozyme is inactivated by urea without any accompanying large change in the secondary and tertiary structures which would be reflected by a change in the intrinsic viscosity and optical rotatory dispersion constants. However, the fine structure of the lysozyme molecule may be changed in over 4 *M* urea, because heat or surface treatment of lysozyme increased the reduced viscosity and area of the monolayer in over 4 *M* urea. No change in the fine structure of the lysozyme molecule, which might be responsible for its inactivation, was detected in the present ex-

periments. An increase in ultraviolet absorption spectrum of lysozyme was observed in urea solutions. However it is difficult to explain the increase

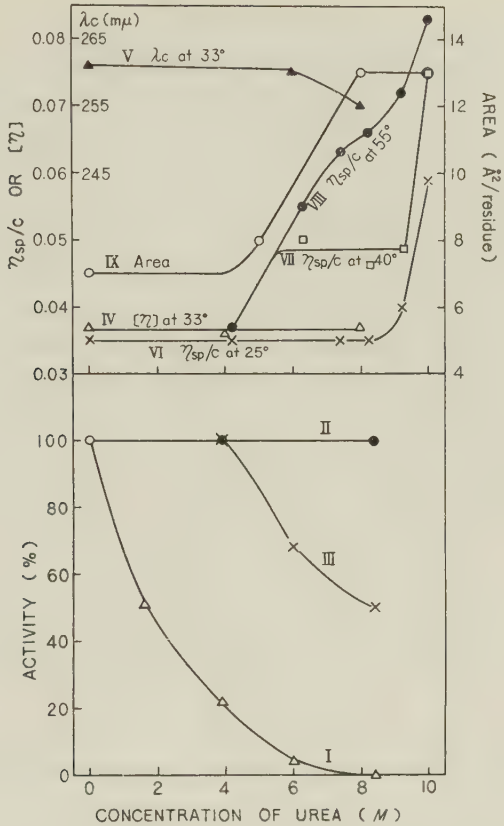

FIG. 2. Effect of urea on activity (*4*), monolayer expansion (*6*, *8*), reduced viscosity (*7*), intrinsic viscosity, and optical rotatory dispersion constants. Curve (I): Activity measured in urea solutions after lysozyme had been preincubated with urea for 1 hour. Curve (II): Activity measured in the absence of urea after lysozyme had been preincubated with urea for 4 hours. Curve (III): The same as curve (II) except that the preincubation period was 25 hours. Curve (IV): Instrinsic viscosity of lysozyme at 33.0°. Curve (V): Optical rotatory dispersion constant of lysozyme at 33°. Curve (VI): Reduced viscosity of lysozyme at 25.0. Curve (VII): Reduced viscosity at 40°. Curve (VIII): Reduced viscosity at 55°. Curve (IX): Relationship between areas of lysozyme monolayers at 2 dynes/cm. and urea concentrations in spreading solution.

in terms of the structural change of lysozyme molecule (*14*). Therefore, the inactivation of lysozyme by urea differs from that of α-chymotrypsin

and trypsin. As shown by Harris (*15*) in the latter cases the change in viscosity was in parallel with inactivation.

SUMMARY

To study the relationship between changes in activity and the physicochemical properties of lysozyme, we measured the intrinsic viscosity and optical rotatory dispersion constants at various concentrations of urea.

The intrinsic viscosity of lysozyme in 8 *M* urea was the same as that of native lysozyme at 33.0°. The dispersion constant of lysozyme in 8 *M* urea was only slightly smaller than that of the native protein.

The inactivation of lysozyme by urea was not in parallel with the change in the viscosity and the dispersion constant at 33°. However, the inactivation and the change in reduced viscosity of lysozyme at higher temperatures were in parallel. It was suggested that a change in fine structure of the lysozyme, which was not reflected by changes in the intrinsic viscosity or dispersion constants, was responsible for its inactivation.

The authors wish to express their hearty thanks to Prof. T. Isemura of the Institute and to Prof. M. Funatsu and Dr. K. Hayashi of the Kyushu University for their encouragements in the present experiments.

REFERENCES

(*1*) Linderstrøm-Lang, K. U., and Schellman, J. A., "The Enzymes," edited by Boyer, P. D., Lardy, H., and Myrbäck, K., Academic press, New York, p. 443 (1959)
(*2*) Léonis, J., *Arch. Biochem. Biophys.*, **65**, 182 (1956)
(*3*) Dickman, S. R., and Procter, C. M., *Arch. Biochem. Biophys.*, **40**, 364 (1952)
(*4*) Hamaguchi, K., Rokkaku, K., Funatsu, M., and Hayashi, K., *J. Biochem.*, **48**, 351 (1960)
(*5*) Glazer, A. N., *Australian J. Chem.*, **12**, 304 (1959)
(*6*) Hamaguchi, K., *J. Biochem.*, **42**, 449 (1955)
(*7*) Hamaguchi, K., *J. Biochem.*, **45**, 79 (1958)
(*8*) Hamaguchi, K., *Bull. Chem. Soc. Japan*, **31**, 123 (1958)
(*9*) Halwer, M., Nutting, G. C., and Brice, B. A., *J. Am. Chem. Soc.*, **73**, 2786 (1951)
(*10*) Yang, J. T., and Foster, J. F., *J. Am. Chem. Soc.*, **77**, 2374 (1955)
(*11*) Jirgensons, B., *Arch. Biochem. Biophys.*, **41**, 333 (1952)
(*12*) Jirgensons, B., and Straumains, L., *Arch. Biochem. Biophys.*, **68**, 319 (1957)
(*13*) Yang, J. T., and Doty, P., *J. Am. Chem., Soc.*, **79**, 761 (1957)
(*14*) Wetlaufer, D. B., Edsall, J. T., and Hollingworth, B. R., *J. Biol. Chem.*, **233**, 1421 (1958)
(*15*) Harris, J. I., *Nature*, **177**, 471 (1956)

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PREPARATION AND SOME PROPERTIES OF RIBONUCLEIC ACID OF YEAST 80S PARTICLE

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(Received for publication, February 16, 1960)

Although some reports have been issued on the preparation (*1–3*) and characterization (*3*, *4*) of ribonucleic acid (RNA) in yeast, the most of them were done on RNA from whole cell which contains various RNA's. To give any evaluation for the properties of RNA, it is desirable to study RNA biologically and physically well-characterized. Current studies have shown that the cytoplasmic ribonucleoprotein particles from various sources exists in relatively homogeneous state in respect to their molecular size (*5–8*) and play the role in protein biosynthesis (*9–12*). Among them, the ribonucleoprotein particle isolated from yeast extract by Chao and Schachman (*5*) has extremely uniform size, and they called it yeast 80S particle. Webster (*13*) suggested its participation in protein synthesis. Recently some reports have been presented on RNA's prepared from ribonucleoprotein particles of animal sources (*14–16*). Present paper deals with preparation and some properties of RNA from yeast 80S particle.

MATERIALS

Yeast—Baker's yeast (*Saccharomyces cerevisiae, strain Kaneka*) was grown in peptone-glucose medium (*17*), harvested during the logarithmic phase and washed with buffer solution for the extraction of particles (see below) to free from the culture medium.

Preparation of 80 S Particle—The preparation of 80 S particle was carried out by the method of Chao and Schachman (*5*) with slight modifications. Yeast cells were ground with quartz sand for 20 minutes and mixture of sand and broken cells was suspended in 0.005 *M* K-phosphate buffer containing 0.001 *M* $MgSO_4$ and 0.75×10^{-3} *M* $CaCl_2$, pH 7.0. The extract was centrifged at first at 2,000 r.p.m. for 20 minutes to remove the sand, intact cells and cell debris, and then centrifuged at 10,000 r.p.m. for 20 minutes. The precipitate was discarded. The supernatant was centrifuged at 40,000 r.p.m. for 60 minutes in a Spinco ultracentrufuge model L, No. 40 rotor. The pellet was resuspended in the same buffer solution and the same process was repeated. In the case of the large scale preparation, particle was spun down at 30,000 r.p.m. for 90 minutes using a Spinco ultracentrifuge model L, No. 30 rotor. All the procedure for the preparation of 80 S particle was carried out below 5°.

METHODS

Sedimentation—Ultracentrifugal analyses were made in a Spinco ultracentrifuge model E using a schlieren optical system. Sedimentation constants were denoted as Svedberg

unit at 20°, $S_{20,w}$,. Except for the case especially mentioned, analyses were done in 0.1 to 0.3 g. per 100 ml. solution of RNA and correction to zero concentration was not made.

Viscosity—Viscosity measurements were made with a Ostwald viscosimeter at 20.0°.

Diffusion—Diffusion measurements were done in a Spinco electrophoresis-diffusion model H using a schliren optical system at 0.5°. Diffusion constants were determined as the value at 20°, $D_{20,w}$,.

Absorbancy—The dependence of extinction coefficient at 258 mμ on the temperature was determined in a Cary spectrophotometer model 14 with the use of cell compartment in which temperature-controlled water circulated from thermostat. Measurements were done after equilibrium value was attained at each temperature.

Optical Rotation—Optical rotation was measured at 589 mμ in a Rudolph photoelectric polarimeter model 200 with the sodium arc as the light source.

Determination of Concentration—The concentration of RNA was determined by the measurement of absorbancy. The extinction coefficient at 258 mμ of the solution containing 1 g. of RNA per 100 ml. of 0.01 *M* K-phosphrte buffer, pH 7.0, is 195.

Chemical Analysis—Phosphorus analysis was made with the method of Allen (*18*). Nitrogen content was determined by micro-Kjeldahl analysis. Protein content was determined by Lorwy's method (*19*) and denoted as crystalline ovalbumin equivalent. Deoxyribonucleic acid was determined by cysteine-H_2SO_4 method (*20*). Determination of relative molar ratio of bases was done as usual. Thus, RNA was hydrolysed with 1 *N* HCl for 1 hour at 100°. The hydrolysate was chromatographed on filter paper (Toyo Roshi No. 51 A) with *tert*-butanol-HCl-water (*21*). Pyrimidine nucleotides and purine bases separated were eluted with 0.1 *N* HCl and the amount of each substance was determined referring to the corresponding molar extinction.

PREPARATION AND RNA

Purified 80S particle was almost homogeneous ultracentrifugally and showed almost the same sedimentation constant as that of Chao and Schachman (*5*) (Fig. 1). Fig. 2 shows the zone electrophoresis pattern

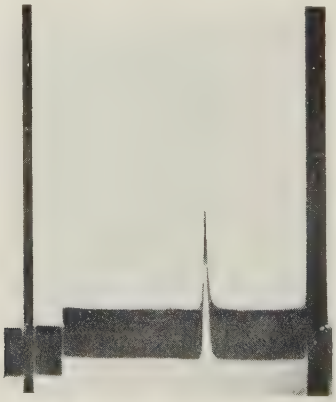

Fig. 1. Ultracentrifuge pattern of yeast 80 S particle in 0.005 *M* K-phosphate buffer containing 0.001 *M* $MgSO_4$ and 0.75×10^{-3} *M* $CaCl_2$, pH 7.0, 12 minutes after reaching speed of 42,040 r.p.m.

on starch. The peak of RNA was almost symmetrical, but a little trailing of protein was observed.

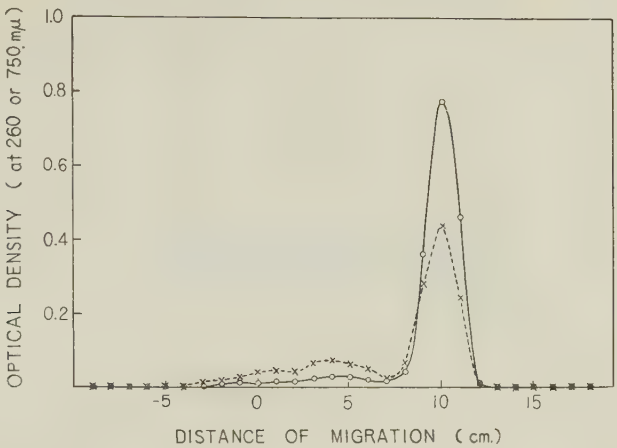

Fig. 2. Zone electrophoresis pattern of yeast 80 S particle on starch.

—○— shows the RNA estimated as the absorbancy at 260 mμ and --×-- shows the protein estimated by the Folin reagent. Determinations were done for the eluate for each 1 cm. segment of starch. Electrophoresis run was carried out at 25 volts per cm. for 10 hours in 0.005 *M* K-phosphate buffer, containing 0.001 *M* $MgSO_4$ and 0.75×10^{-3} *M* CaCl.

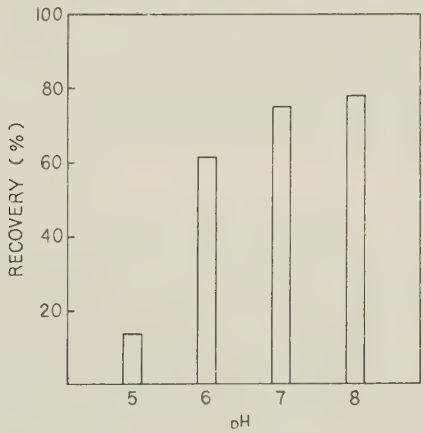

Fig. 3. Recovery of RNA in the aqueous phase after the separation of phenol mixture of various pH.

80 S particle in dilute buffer solution was mixed with phenol. The mixture was stirred for 1 hour and adjusted the pH with addition of HCl or KOH. Aqueous phase was separated by centrifugation at 10,000 r.p.m. for 10 minutes. RNA released in the aqueous phase from the phenol mixture of various pH was determined and denoted as the percentage of RNA in 80 S particle.

The preparation of RNA from 80S particle was carried out by the modification of Kirby's phenol method (*22*). 80S particle in 0.005 *M* K-phosphate buffer containing 1.0×10^{-3} *M* $MgSO_4$ and 0.75×10^{-3} *M* $CaCl_2$, pH 7.0, was mixed with the equal volume of redistilled phenol (90 per cent v/v) and stirred for 1 hour. At this step the pH of the mixture is important for the good recovery of RNA. As shown in Fig. 3, recovery of RNA in the aqueous phase depends remarkably on the pH of the mixture. The more the pH of the mixture was increased, the more the RNA released in the aqueous phase increased. As the pH of the mixture of phenol and solution in dilute buffer is always about 5, it is required for the good recovery of RNA to raise the pH of the mixture. So the mixture was immediately titrated to pH 7.0 by dropwise addition of 1 *N* KOH. After standing for 10 minutes in ice, the mixture was centrifuged for 10 minutes at 10,000 r.p.m. to separate phenol and aqueous phases. The turbid aqueous phase was pipetted out. After washing the residual phenol with 0.01 *M* K-phosphate buffer, pH 7.0, the aqueous phase was separated by the same manner. The aqueous phases were combined and centrifuged for 20 minutes at 10,000 r.p.m.. The phenol further separated was discarded. To the aqueous solution containing RNA mixed with potassium acetate to final concentration of 2 per cent were added two volumes of cold ethanol adjusting pH to 7.0 by the addition of 1 *N* HCl. After 10 minutes, the precipitate was collected by centrifugation. The precipitate was redissolved in 0.01 *M* K-phosphate buffer, pH 7.0. Ethanol precipitation and redissolution in buffer solution were repeated twice and finally the RNA solution was centrifuged at $100{,}000\times g$ for 10 minutes to eliminate the insoluble matter. The supernatant was dialyzed for 3 to 4 hours against 0.01 *M* K-phosphate buffer, pH 7.0. The amount of RNA finally obtained was 75 to 80 per cent of that found in original particle. All the procedure was carried out below 5°.

SOME PROPERTIES OF RNA

Sedimentation pattern of RNA in 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, was shown in Fig. 4. It gave two boundaries with trace amount of RNA of slowly sedimenting component. Fig. 5 shows the dependence of sedimentation constant on the concentration of RNA. Although the determinations were done only in three points, each plot fitted well to the the straight line. So extrapolation to zero concentration was made and sedimentation constants were determined as 28S and 19S, respectively. The ratio of the amount of 28S component to 19S component was about 3 judging from the area under the peak.

When the preparative procedure was carried out by the method of Gierer and Schramm (*23*), that is shaking with phenol for 8 minutes and removal of phenol with ether, in which all the procedure was completed in 1 hour, the same sedimentation boundaries and velocities were obtained.

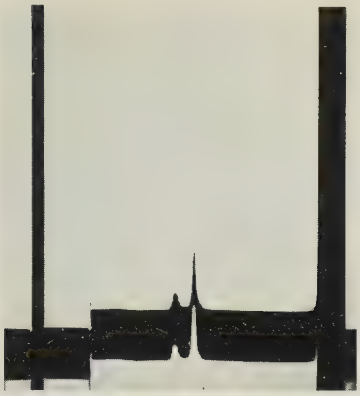

FIG. 4. Ultracentrifuge pattern of RNA prepared from yeast 80 S particle.

0.76 g. per 100 ml. of 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, 16 minutes after reaching speed of 52,420 r.p.m.

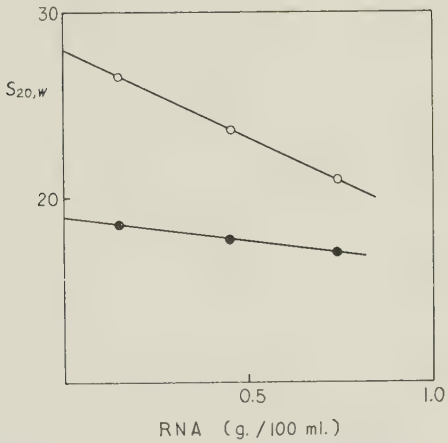

FIG. 5. The dependence of sedimentation constant on the concetration of RNA in 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0.

—○— shows the fast sedimenting component and —●— the following one.

Viscosity data are shown in Fig. 6. $\eta_{sp/c}$ in 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, practically did not depend on the concentration of the solution. Intrinsic viscosity of RNA in saline was determined as 0.55 dl./g.. On the contrary, RNA solution dialyzed for 24 hours against distilled water with frequent change of external water showed high viscosity and its $\eta_{sp/c}$ increased with the decrease of the concentration of RNA.

Diffusion experiments were carried out on the RNA solution of 0.57

and 0.31 g. per 100 ml. of 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, and diffusion constants calculated by the moment method were

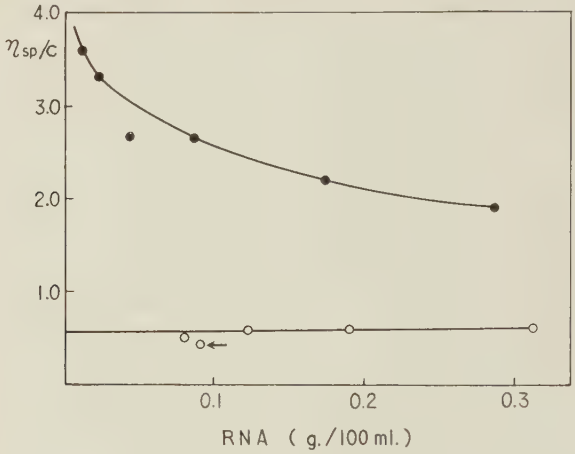

Fig. 6. The dependence of $\eta_{sp/c}$ of RNA of yeast 80 S particle on the concentration.

—○— shows the viscosity of RNA in 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, —●— shows the viscosity of RNA dialyzed against distilled water for 24 hours and arrow shows the viscosity of water dialyzed RNA in saline. Measurements were made at 20.0°.

1.24×10^{-7} and 1.05×10^{-7} cm.^{2}/sec., respectively. Assuming diffusion constant to increase linearly with the rise of concentration of RNA, extrapolated value to zero concentration, $D^0_{20,w}$, was estimated as 0.82×10^{-7}cm^2./sec..

$[\alpha]_{589}$ in 0.1*M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, was 190° at 11° and that of alkaline hydrolysate neutralized with HCl was 20°.

Fig. 7a shows the sedimentation pattern of the RNA which was obtained

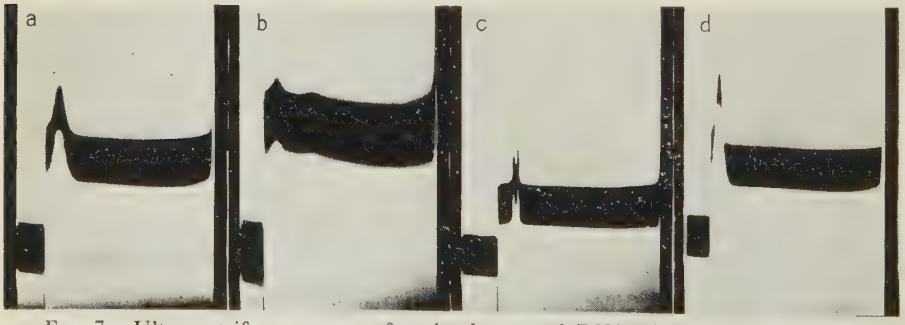

Fig. 7. Ultracentrifuge pattern of variously treated RNA of yeast 80 S particle. (a), RNA prepared by phenol at 80° for 10 minutes. (b), RNA prepared by phenol at 40° for 30 minutes. (c), RNA standing in cold in the diluted state (0.1 g. per 100 ml.) and then collected by ethanol precipitation. (d), RNA dialyzed overnight against 0.001 *M* EDTA in 0.01*M* K-phosphate buffer, pH 7.0. All the run was done on the RNA of 0.1 to 0.3 g. per 100 ml. of 0.01 *M* K-phosphate buffer, pH 7.0, 8 minutes after reaching speed of 59,780 r.p.m.

by the treatment of 80S particle with phenol at 80° for 10 minutes. In this case, fast sedimenting component completely disappeared and relatively broad peak was observed in the range of 5S*. When phenol treatment was carried out at 40° for 30 minutes, the existence of 23S component was observed with 5S one (Fig. 7b).

The dependence of the optical density on temperature was determined on RNA in 0.01 *M* K-phosphate buffer, pH 7.0, and the results are shown in Fig. 8. The steep increase of optical density was observed between 30° and 40°. After rising the temperature to 76°, measurements were done in

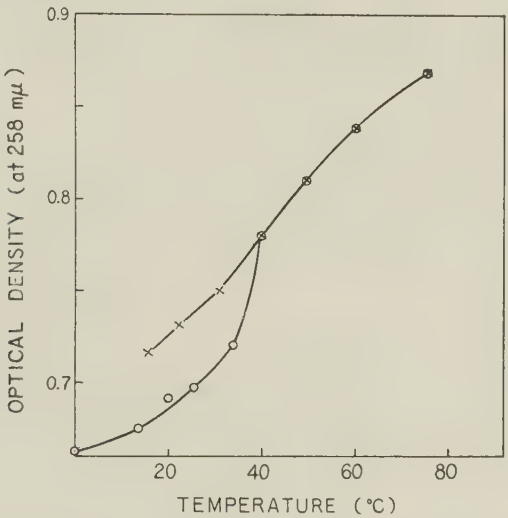

FIG. 8. The dependence of optical density at 258 mμ of RNA of yeast 80 S particle in 0.01 *M* K-phoshate buffer, pH 7.0, on temperature.

—○— shows in the course of rising the temperature and —×— shows in the course of lowering temperature.

decreasing temperature. Optical density below 40° was shown to be consistently higher than the initial value. These results show that the irreversible process caused by temperature occurs around 40°.

In the course of these studies some aspects on the stability of the RNA were gained. Even if the RNA was dialyzed against 0.01 *M* K-phosphate buffer containing 0.001 *M* $MgSO_4$, pH 7.0, overnight in the cold, or standing at 0.5° for 4 days in 0.1 *M* KCl containing 0.01 *M* K-phosphate buffer, pH 7.0, the decrease of sedimentation constant was not observed practically. But dialysis against distilled water sometimes resulted in the fall of sedi-

* In the following experiments, as the sedimentation constants were measured for the solution in 0.01 *M* K-phosphate buffer, direct comparison is not possible due to the charge effect anticipated in such a low ionic strength. The value of 23 S obtained on "standard RNA" (0.1 g. per 100 ml.) in 0.01 *M* K-phosphate buffer may be compared with following data.

mentation velocities, especially in the diluted state, 0.1 g. per 100 ml. 14S and 11S were gained (Fig. 7c). Dialysis against 0.001 *M* ethylenediaminetetra-acetic acid (EDTA) in 0.01 *M* K-phosphate buffer (pH 7.0) caused profound decrease of sedimentation constants. Fast sedimenting component completely disappeared and relatively broad peak having about 5S appeared (Fig. 7d). Its boundarry shape and velocity in sedimenting run was similar to that of RNA prepared with phenol at 80° (see Fig. 7a).

The results of chemical analysis are tabulated in Table I. Phosphorus

TABLE I

Chemical Composition of RNA from Yeast 80 S Particle

Adenylic acid moles per 100 moles of nucleotides	26.8
Guanylic acid moles per 100 moles of nucleotides	27.9
Cytidylic acid moles per 100 moles of nucleotides	19.2
Uridylic acid moles per 100 moles of nucleotides	26.1
phosphorus g. per 100 g. of dry weight	7.7 (7.64)
nitrogen g. per 100 g. of dry weight	12.3 (12.5)
DNA	not detectable
protein g. per 100 g. of dry weight	below 4.2

The number in parenthesis means the calculated value from the nucleotide composition regarding the RNA as potassium salt.

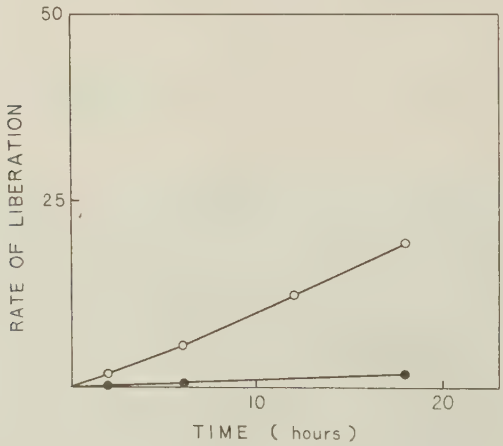

FIG. 9. Liberation of acid soluble nucleotides during the incubation of RNA of of yeast 80 S particle in 0.01 *M* K-phosphate buffer.

0.3 g. of RNA in 100 ml. of 0.01 *M* K-phosphate buffer, pH 7.0 was incubated in the presence (—○—) and in the absence (—●—) of 0.01 *M* EDTA, After the various time intervals a portion of the sample was taken up and mixed with equal volume of 1.0 *N* HCl. Precipitate was removed by centrifugation and supernatant was diluted 5 times with water to read the optical density at 260 mμ. The rate of liberation was denoted as the percentage of its absorbancy to that of alkaline hydrolysate.

and nitrogen contents show quite coincidence with that of calculated value from the nucleotide composition. As shown in Table I, RNA preparation contains below 4 per cent of protein, it seems possible to include trace of ribonuclease. RNA solution was incubated at 37° and after various intervals, a portion of solution was taken up and mixed with the equal volume of 1 *N* HCl in the cold. The precipitate was spun down and the supernatant was diluted to final concentration of HCl of 0.1 *N*. Its absorbancy at 260 mμ was measured (Fig. 9). In the absence of EDTA, the liberation of acid soluble nucleotides was dot practically observed until 12 hours, but in the presence of EDTA, the remarkable liberation of them was observed. Pre-incubation with the equal weight of pronase (proteinase from *Streptomyces griseus*) at 37° for 30 minutes lowered the rate of them (Table II), so the

TABLE II

Changes of the Rate of Liberation of Acid Soluble Nucleotides from RNA of Yeast 80S Particle by Various Treatments

Trertment	0 hours %	12 hours %
Incubated with pronase at 37° for 30 min.	3.0	1.0
Heated at 80° for 10 min.	1.0	19.5
Non	0	0

RNA treated was incubated with 0.01 *M* EDTA in 0.01 *M* K-phosphate buffer, pH 7.0 at 37° for 12 hours.

The rate of liberation was denoted as percentage of the acid soluble nucleotides to all the nucleotide.

liberation of acid soluble nucleotides may be regarded to be caused by the contamination of ribonuclease. RNA prepared with phenol at 80° still liberated soluble nucleotides in the presence of EDTA. Indeed, its activity was not destroyed by incubation at 80° for 10 minutes (Table II).

DISCUSSION

RNA prepared from yeast 80S particle by phenol at low temperature has extremely high sedimentation constant comparing with other preparations of RNA from yeast (*3*, *4*), and relatively uniform size even though a part of sharpness of sedimentation pattern may be caused by the hypersharp effect. Although it is difficult to give the exact model of the molecule at present, some estimation for it can be made from the present data. The specific rotation of the RNA is similar to that of tobacco mosaic virus RNA (*24*) at room temperature. Current studies show that a portion of the optical rotation of macromolecule reflects the helical content (*25*). In view of these considerations Doty *et al.* considered (*24*) that the specific rotation of 180° at 589 mμ of tobacco mosaic virus RNA corresponds to 40 to 60 per cent of helix content by comparing with absorbancy at 258 mμ

depending on temperature. So it is supposed that RNA of yeast 80S particle consists of helical region and random coil.

On the other hand, RNA in water shows higher viscosity than in electrolytes. This behavior is characteristic of flexibly coiled synthetic polyelectrolytes (*26*). But in the preliminary experiments RNA dialyzed against EDTA showed much higher viscosity in water. This fact gives room that metal may hold some parts of RNA in the rigid form. The detailed feature is now under study. From the above consideration the RNA is described as a molecule which consists of the relatively rigid helical part and the flexible random coil structure.

For the configurational properties, Flanklin *et al.* suggested (*27*) in the studies of X-ray diffraction diagram that isolated RNA from 80S particle is not similar to that in the particle in which RNA might exists in helical structure. RNA isolated in the present manner may have some helical region. It can be considered that some configurational properties of RNA in particles was kept during the preparative procedure.

For the determination of the molecular weight, the present experiments gives only tentative estimation, largely due to multicomponent system. As 28S component occupies prevailing amount, the calculation was made on this component assuming that apparent viscosity and diffusion data of this preparation largely reflect those of 28S component. 2.0×10^6 was gained from the sedimentation and diffusion data with few assumption, except for the use of partial specific volume of 0.58 (*4*). On the other hand, assuming the RNA to be flexible random coil, the molecular weight was calculated by Mandelkern-Flory equation (*28*),

$$M^{3/2}=\frac{s_0\cdot[\eta]^{1/3}\cdot\eta_0 N}{\Phi^{1/3}\,P^{-1}(1-V\rho)}$$

where $\Phi^{1/3}P^{-1}$ was given by them to be 2.5×10^6. From the value of sedimentation constant and intrinsic viscosity, the molecular weight was obtained as 1.5×10^6.

Chao and Schachman (*5*) estimated the molecular weight of 80S particle to be 4.1×10^6. Along with the RNA content in this particle of 43 per cent, it was suggested that the maximum molecular weight of RNA is 1.7×10^6 (*29*). Although the molecular weight obtained here is tentative one, it is not so far from the expected value assuming one particle contains one molecule of RNA. The RNA prepared in the present manner can be estimated as native one in regards to the molecular size.

Hall and Doty (*16*) found in temperature-optical density profile that RNA melts at critical temperature by disruption of hydrogen bonds. In the present case, RNA melts around 40°, and even if cooled to room temperature it does not return reversibly. Indeed, RNA treated above 40° completely dissociated to smaller units which have relatively uniform size. Moreover, at this temperature, the coexistence of intact and dissociated RNA was observed. In the range of low temperature, EDTA caused such a dissociation. In spite of the presence of ribonuclease which acts in the

presence of EDTA, it is conjected that such a dissociation may be caused by disruption of linkage which connects the basal sub-units rather than by random hydrolysis by ribonuclease. At any rate, RNA prepared by the detergent from yeast described by Crestfield *et al.* (*3*) is supposed to be a sub-unit RNA and it is emphasized that the preparation must be carried out in the temperature below 40° and in the absence of EDTA for the characterization of native RNA.

RNA of 80S particle is considerably homogeneous in its size with the exception of accompanying 19S component. The latter component was frequently observed in phenol RNA from other sources (*14*, *16*, *30*). Whether it is in a intact state or partially degradaded one cannot yet be determined. From the fact that prolonged dialysis against distilled water causes the increase of the second component, the latter explanation is plausible.

From the present results RNA may be supposed to be uniform in size, though the compositional heterogeneity is not known. But its possibility exists from the following reason. The ratio of release of RNA in aqueous phase followed by phenol treatment much depends on pH. Recently Sibatani *et al.* (*31*) found that nucleotide composition and metabolic activity differ from each other significantly between aqueous and phenol phase. These facts suggest that compositional heterogeneity might exists in the uniform size RNA of yeast 80S particle.

SUMMARY

1. A method of preparation of RNA from yeast 80S particle which gives high yield and consistently high molecular weight was described. The pH and temperature are especially important.

2. RNA prepared in such a manner has the value of 28S along with 19S in sedimentation constant, 0.55 dl./g. in intrinsic viscosity and 0.82×10^{-7} cm.^{2}/sec. in diffusion constant.

3. RNA dissociates irreversibly into small sub-units (about 5S) above 40° or in the presence of EDTA in low temperature. The significant change in optical density occured around 40°.

4. RNA prepared by phenol method still contains ribonuclease and liberated acid soluble nucleotides especially in the presence of EDTA.

5. From the viscosity behavior and the specific rotation of 190° at 589 mμ, configurational properties of RNA was discussed.

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REFERENCES

(*1*) Holden, M., and Pirie, N. W., *Biochem. J.*, **60**, 46 (1955)
(*2*) Hakim, A. M., *J. Biol. Chem.*, **225**, 689 (1957)
(*3*) Crestfield, A. M., Smith, K. C., and Allen, F. W., *J. Biol. Chem.*, **216**, 185 (1955)
(*4*) Watanabe, I., and Iso, K., *J. Am. Chem. Soc.*, **72**, 4836 (1950)
(*5*) Chao, F. C., and Schachman, H. K., *Arch. Biochem. Biophys.*, **61**, 220 (1956)
(*6*) Peterman, M. L., and Hamilton, M. G., *J. Biol. Chem.*, **224**, 725 (1957)
(*7*) Tissieres, A., and Watson, J. D., *Nature*, **182**, 778 (1958)
(*8*) Gillchriest, W. C., and Bock, R. M., "Microsomal Particles and Protein Synthesis", Pergamon Press, New York, p. 1 (1958)
(*9*) Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C., *J. Biol. Chem.*, **217**, 111 (1955)
(*10*) Balis, M. E., Samouth, K. D., Hamilton, M. G., and Peterman, M. L., *J. Biol. Chem.*, **233**, 1152 (1958)
(*11*) Siekevitz, P., and Palade, G. E., *Federation Proc.*, **18**, 324 (1958)
(*12*) Schweet, R., Lamform, H., and Allen, E., *Proc. Natl. Acad. Sci. U. S.*, **44**, 1029 (1958)
(*13*) Webster, G. C., *J. Biol. Chem.*, **229**, 535 (1957)
(*14*) Gierer, A., *Z. Naturforsch.*, **13b**, 788 (1958)
(*15*) Takanami, M., *Biochim. et Biophys. Acta*, **29**, 430 (1958)
(*16*) Hall, B. D., and Doty, P., *J. Mol. Biol.*, **1**, 111 (1959)
(*17*) Halvorson, H., and Spiegelman, S., *J. Bacteriol.*, **64**, 207 (1952)
(*18*) Allen, F. W., *Biochem. J.*, **34**, 858 (1940)
(*19*) Lowry, O. H., Rosenbrugh, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)
(*20*) Stumpf, P., *J. Biol. Chem.*, **169**, 367 (1951)
(*21*) Smith, J. D., and Markham, R., *Biochem. J.*, **46**, 509 (1950)
(*22*) Kirby, K. S., *Biochem. J.*, **64**, 405 (1956)
(*23*) Gierer, A., and Schramm, G., *Z. Naturforsch.*, **11b**, 139 (1956)
(*24*) Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R., and Litt, M., *Proc. Natl. Acad. Sci., U. S.*, **45**, 482 (1959)
(*25*) Yang, J. T., and Doty, P., *J. Am. Chem. Soc.*, **79**, 761 (1957)
(*26*) Fuoss, R. M., *Discussions Faraday Soc.*, **11**, 125 (1951)
(*27*) Flanklin, R. E., Klug, A., Finch, J. T., and Holmes, K. C., *Discussions Faraday Soc.*, **25**, 197 (1958)
(*28*) Mandelkern, L., and Flory, P. J., *J. Chem. Phys.*, **20**, 212 (1952)
(*29*) Cheng, P. Y., *Nature*, **179**, 426 (1957)
(*30*) Littauer, U. Z., and Eisenberg, H., *Biochim. et Biophys. Acta*, **32**, 320 (1959)
(*31*) Sibatani, A., Yamana, K., Kimura, K., and Okagaki, H., *Biochim. et Biophys. Acta*, **33**, 590 (1959)

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TURNIP PEROXIDASE

V. THE EFFECT OF SEVERAL CHEMICAL REAGENTS UPON THE ACTIVITY OF TURNIP PEROXIDASE D

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On the basis of kinetic studies on the effect of hydrogen ion concentration upon the activity of turnip peroxidase D, it was inferred previously that an α-ammonium group in the protein moiety may play an essential role in the reaction of Complex II with hydrogen donor molecules (*1*).

The experiments in the present paper were performed to obtain further information as to the nature of an essential group in the protein moiety of the enzyme by investigating the effect of various substances which were known to affect specifically a definite group in protein molecules.

MATERIALS AND METHODS

The enzyme used throughout this work was turnip peroxidase D prepared as described previously (*2*). The method by which the enzyme was treated with chemical reagents is described below in the reports on the individual experiments. The activity of the enzyme modified by chemical reagents was estimated by the method described previously, determining the value of k_1 (the rate constant of the reaction of formation of Complex I) and that of k_d* (the rate constant of the reaction of Complex II with hydrogen donor) (*1*). The reaction mixture for the determination of k_1 consisted of hydrogen peroxide, $3.6\times10^{-5}\,M$; guaiacol, $1.0\times10^{-3}\,M$; phosphate buffer, pH 5.8, $3.2\times10^{-2}\,M$; and 0.1ml. of enzyme as indicated, in a total volume of 3.13 ml. The reaction mixture for the determination of k_d contained hydrogen peroxide, $2.2\times10^{-4}M$; guaiacol, $1.8\times10^{-5}M$; others being the same as in the former experiment.

RESULTS

Effect of Acetic Anhydride—Acetic anhydride is known to be a superior reagent for acylating amino groups of proteins, since it reacts with amino

* As described previously (*1*), the notation "k_d" is used for convenience and does not mean the rate constant in the usual sense, but only means the resultant of several rate constants. However, it can be approximately regarded as k_4 or k_0/K_M assuming Mechanism A or B, respectively, in other words, a constant concerning the rate of reaction between Complex II and hydrogen donor (*1*). For the purpose of the present work, it is enough to investigate the effect of chemical reagents on the value of k_d.

groups in a relatively specific manner and is scarcely harmful to native proteins (*3*). As described in the legend of Fig. 1, the enzyme was treated with acetic anhydride for various time periods, taking into account the conditions described by Fraenkel-Conrat (*4*), and subjected to the measurement of enzyme activity. Fig. 1 shows a typical example of the experimental results. The relative value of k_1 and that of k_d are plotted against the reaction time, during which the enzyme had been in contact

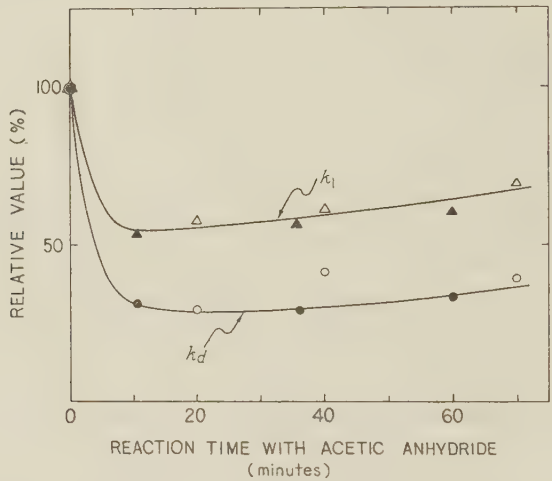

FIG. 1 Effect of acetic anhydride upon the activity of turnip peroxidase D

To 0.4 ml. of the enzyme solution ($3.0 \times 10^{-4} M$) was added 0.4 ml. of saturated solution of sodium acetate, and the mixture was cooled in an ice bath and 10 μl. of acetic anhydride added while stirring. Then, 0.1 ml. each of the mixture was taken out at successive intervals and poured into 400 ml. of water to stop the action of the reagent*. Without delay, the activity of the enzyme in the diluted solution was measured using 0.1 ml. of the diluted solution by the method described in the text. The relative value of k_1 and k_d obtained are plotted as open triangles and circles, respectively, against the reaction time, during which enzyme was in contact with the reagent. Solid triangles and circles mean the relative value of the rate constants obtained when another 10 μl. of acetic anhydride was added at ten minutes after the initial addition and the same method as described above was applied.

* It is reasonable to consider that this dilution actually stops the action of acetic anhydride on the enzyme and that the extremely small quantity of the reagent which comes into the reaction mixture for the determination of k_1 and k_d, has no effect for the measurement, since the rate of the reaction of the sample at zero time agreed with the rate in the absence of the reagent. The same was true in the case of the other reagents described below.

with acetic anhydride. As may be seen from this figure, k_1 and k_d decreased rapidly within ten minutes, but they remained constant thereafter, at levels of 60 and 30 per cent of the initial values, respectively*.

The absorption spectrum of the enzyme was unaffected by the treatment with acetic anhydride, except a slight decrease in the absorbance (90 per cent of the initial value remaining after 80 minutes from the moment of the introduction of the reagent). The fact indicates that the reagent has little influence on the hematin part of the enzyme.

Effect of Nitrite—It is known that nitrous acid reacts not only with aliphatic amino groups to deaminate, but also with sulfhydryl, or phenolic group of proteins (*3*, *5*). However, in the presence of excess nitrous acid

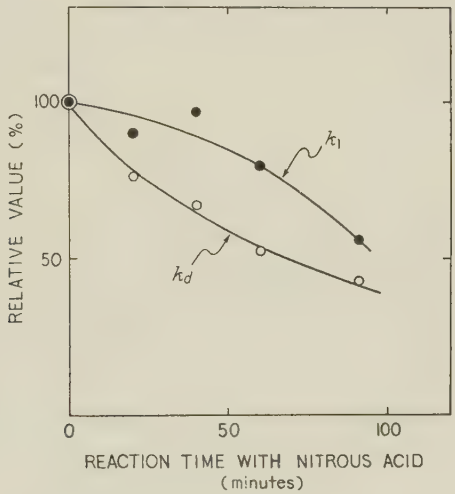

FIG. 2 Effect of nitrous acid upon the activity of turnip peroxidase D

To 0.2 ml. of the enzyme solution (2.6×10^{-4} *M*) was added 1.0 ml. of acetate buffer (0.1 *M*, pH 4.1). The action of nitrous acid upon the enzyme was initiated by the addition of 0.2 ml. of sodium nitrite of 1.3 *M* at 25°, and 0.1 ml. each of the mixture was taken out at successive intervals and poured into 200 ml. of water to stop the action of the reagent. Then, the activity of the enzyme in the diluted solution was measured by the method described in the text. The relative values of k_1 and k_d obtained are plotted as solid circles and open ones, respectively, against the reaction time, during which the enzyme was in contact with nitrous acid.

* That perfect inhibition was not found should not be ascribed to the insufficiency of the amount of the reagent used in this experiment, since the addition of another 10 μl. of acetic anhydride at ten minutes after the first addition of the reagent revealed no change in the values of k_1 and k_d as shown in Fig. 1.

the deamination proceeds much more rapidly than the reaction with phenolic group (*6*). Free sulfhydryl group is not present in the enzyme as described below. Therefore, it is reasonable to consider that nitrous acid attacks only amino groups of the protein moiety of turnip peroxidase D under the present conditions (Fig. 2). The result illustrated in Fig. 2 shows that both k_1 and k_d decreases with the reaction time, the former more slowly than the latter.

Effect of 2,4-Dinitrobenzene Sulfonic Acid—This reagent is also known to react with amino groups of proteins (*7*). The enzyme was treated with the reagent under the conditions described in the legend of Fig. 3. The measurement of enzymatic activity showed that k_1 and k_d decreased together with the reaction time (Fig. 3).

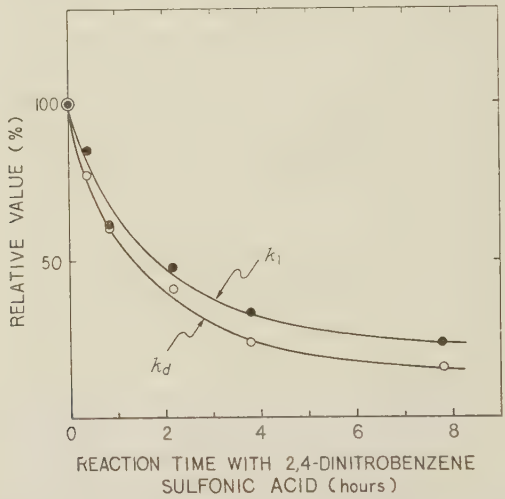

FIG. 3 Effect of 2, 4-dinitrobenzene sulfonic acid upon the activity of turnip peroxidase D

To 0.2 ml. of the enzyme solution (4.6×10^{-4} *M*) was added 1.0 ml. of Na_2CO_3-$NaHCO_3$ buffer (0.2 *M*, pH 10). The mixture was cooled in an ice bath and 0.5 ml. of 2, 4-dinitrobenzene sulfonic acid (0.15 *M*) added. 0.1 ml. each of the mixture was taken out at successive intervals and poured into 200 ml. of water to stop the action of the reagent. Then, the activity of the enzyme in the diluted solution was measured by the method described in the text. The relative values of k_1 and k_d obtained are plotted as solid circles and open ones, respectively, against the reaction time, during which the enzyme was treated with the reagent.

Effect of p-Chloromercuribenzoate—*p*-Chloromercuribenzoate did not inhibit the peroxidase reaction at the final concentration of 2.9×10^{-4} *M*. (The reagent caused the enzyme to precipitate at the final concentration of 2.9×10^{-3} *M*). This follows naturally since free sulfhydryl group could not be

detected by the present author through the spectrophotometric procedure described by Fraenkel-Conrat (*4*).

Effect of p-Diazobenzene Sulfonic Acid—*p*-Diazobenzene sulfonic acid is a representative reagent coupling with imidazole and phenolic groups of proteins. The enzyme was treated with the reagent under the conditions described by Fraenkel-Conrat (*4*), but no change in the activity was found, indicating that the imidazole and phenolic groups of the enzyme protein are not essential for the peroxidase activity.

Effect of Iodine—Iodination of the enzyme protein was carried out in 0.2 *M* Na_2CO_3-$NaHCO_3$ buffer of pH 9.5, by the addition of varying amounts of iodine solution (0.005 *M* I_2 in 0.5 *M* KI) at 0° (*4*). When 3 moles of iodine was used per one mole of the enzyme, no change of k_1 and k_d was found in the course of the reaction during 21 hours. However, when 15 moles of iodine was used per one mole of the enzyme, the values of k_1 and k_d decreased gradually to 58 and 53 per cent of the initial values, respectively.

DISCUSSION

The experiments described above have shown that the reagents which are known to react specifically with amino groups of proteins, namely acetic anhydride, nitrous acid, and 2,4-dinitrobenzene sulfonic acid, diminish the value of k_d of turnip peroxidase D. The fact supports the view set forth previously on the basis of kinetic studies that a primary amino group of the protein moiety is essential for the reaction of Complex II with hydrogen donor. However, the finding that acetic anhydride seems to be unable to inhibit the reaction completely even if a sufficient quantity of the reagent has been used is difficult to be accounted for. Further investigation must be performed on the relation between the degree of acetylation of amino groups and the activity of the enzyme.

The reagents mentioned above also diminished more or less the value of k_1. The fact appears to be inconsistent with the view obtained from the kinetic studies that amino groups are not essential for the formation reaction of Complex I (*1*). However, the discrepancy is explained by assuming that the dinitrophenyl and acetyl groups attached to the amino group* which might be situated rather near to the hematin prevent the approach of hydrogen peroxide to the iron atom of the hematin by the steric hindrance of these groups, especially of the dinitrophenyl group. The relatively slow decrease of k_1 in the case of the treatment with nitrous acid may support the view, since nitrous acid acts as a deaminating reagent and will not prevent the approach of hydrogen peroxide to the hematin. In this connection, it is interesting to note that the hematin of horseradish peroxidase was suggested to be somewhat buried in the protein moiety (*8*).

* The amino group under consideration is not necessarily the essential group described previously (*1*).

In addition to the three reagents mentioned above, iodine also showed a slight inhibitory effect. However, the effect of iodine is somewhat complicated, since iodine attacks not only sulfhydryl, phenolic, and imidazole groups, but also causes certain oxidative reaction (*4*). In view of the evidence of the absence of sulfhydryl group and the experimental result on the effect of diazobenzene sulfonic acid, the effect of iodine observed in the present experiment may be due to complicated oxidative reactions or denaturation of the protein. Actually, the enzyme was found to become insoluble after the treatment with iodine for one day followed by dialysis.

SUMMARY

1. When turnip peroxidase D was treated with acetic anhydride, nitrous acid, and 2, 4-dinitrobenzene sulfonic acid, the activity of the enzyme was found to decrease remarkably, rather owing to the decrease of the rate of reaction of Complex II with hydrogen donor. This finding seems to support the view set forth previously from kinetic studies that a certain amino group of the protein moiety of the enzyme may play an essential role in the reaction of Complex II with hydrogen donor. The rate of the formation of Complex I, however, also decreased more or less, especially in the case of the treatment with 2, 4-dinitrobenzene sulfonic acid. A possible cause of the decrease was discussed.

2. The absence of free sulfhydryl group in the protein moiety of the enzyme was confirmed by kinetic and spectrophotometric studies.

3. Diazobenzene sulfonic acid did not cause any loss of the activity of the enzyme. Iodine showed a slight inhibitory effect, which seemed, however, to be due to the denaturation of the enzyme caused by the reagent. From these results, it may be inferred that imidazole and phenolic groups of the protein moiety are not essential for the activity of the enzyme.

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REFERENCES

(*1*) Hosoya, T., *J. Biochem.*, **48**, 185 (1960)
(*2*) Hosoya, T., *J. Biochem.*, **47**, 369 (1960)
(*3*) Putnam, F. W., "The Proteins" edited by Neurath, H., and Bailey, K., published by the Academic Press Inc., New York, Vol. **I**, part B, p. 893 (1953)
(*4*) Fraenkel-Conrat, H., "Methods in Enzymology" edited by Colowick, S. P., and Kaplan, N. O., published by the Academic Press Inc., Vol. **IV**, p. 247 (1957)
(*5*) Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Rev.*, **41**, 151 (1947)
(*6*) Philpot, J. S. L., and Small, P. A., *Biochem. J.*, **32**, 542 (1938)

(*7*) Minakami, S., Titani, K., Ishikura, H., and Takahashi, K., "Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957" published by the Maruzen, Tokyo, p. 211 (1958)

(*8*) Granik, S., and Gilder, H., *Advances in Enzymol.*, **7**, 305 (1947)

The Journal of Biochemistry, Vol. 48, No. 3, 1960

STUDIES ON AN AMYLASE OF CANDIDA TROPICALIS VAR. JAPONICA

II. FURTHER EVIDENCES FOR BROAD SPECIFICITY OF THE AMYLASE

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Experiments reported previously (*1*) suggested that the cells of *Candida tropicalis* var. *japonica* contain an amylase of unusually broad specificity, capable of hydrolyzing maltose and hetero α-glucosides as well as soluble starch and capable also of transferring glucose from α-phenyl glucoside to glycerol. Ratios among these various activities remained essentially constant after subjecting enzyme preparations to treatment with heat, acid or alkali, or to fractionation by paper electrophoresis or on ion exchange resin.

The present experiments confirm and extend the concept that *Candida* amylase has a wide range of action. Strong support for the idea that all of the described activities are the attributes of a single enzyme has been obtained both from experiments involving zone electrophoresis, and from studies of inhibiton.

EXPERIMENTAL AND RESULT

Hydrolysis of Isomaltose and Limit Dextrins by the Candida Enzyme—Several authors have reported that mold enzymes capable of producing glucose from starch also are able to hydrolyze α- and β-amylase limit dextrins (*2–5*). In preliminary experiments *Candida* amylase, which produces glucose from starch, was also found able to attack residues resistant to α- and β-amylases, as well as isomaltose. The following experiments illustrate these actions, and provide data permitting their inclusion in the electrophoretic examination of *Candida* amylase.

The α-amylase limit dextrin was prepared from glutinous rice starch by first treating the starch with a purified bacterial α-amylase (Nagase Co.), followed by two overnight treatments at 37° with saliva. From the boiled and concentrated digest, the α-limit dextrin was separated as a fraction between 60 and 95 per cent by volume ethanol. Reducing value by Somogyi's method (*6*) indicated an average degree of polymerization of 7. The β-amylase limit dextrin was prepared from Lintnerized glutinous rice starch by two successive overnight treatments at 37° with wheat β-amylase, obtained

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according to the method of Meyer *et al.* (*9*). This material was used instead of the high molecular weight β-amylase limit dextrin of native glutinous rice starch because it was known from unpublished experiments that native starch is less rapidly degraded by *Candida* amylase than partly hydrolyzed products such as soluble starch. Isomaltose was kindly supplied by Dr. Shibasaki of Tohoku University.

For determination of pH-activity curves (Fig. 1), mixtures were prepared consisting of 1 ml. of substrate (0.004 *M* isomaltose, 0.5 per cent α-amylase limit dextrin, or 1.0 per cent β-amylase limit dextrin), 0.5 ml. McIlvaine buffer (pH range from 2.4 to 7.4), and 0.5 ml. of resin treated *Candida* enzyme solution (*1*). The latter contained (for mixtures with isomaltose, α- and β-dextrin respectively) 400, 220 or 150 units per ml. of enzyme solution based on capacity to hydrolyze Yashima Co. soluble starch (*1*). All tubes were incubated at 37° for 1.5 hours (isomaltose). 16 hours (α-dextrin), or 44 hours (β-dextrin). After incubation, 5 ml. of 1 per cent sodium carbonate solution was added to each tube, and the reducing power was determined by Somogyi's method (*6*). After correction for the reducing values of control mixtures in which the enzyme was omitted, the per cent hydrolysis was calculated for each substrate.

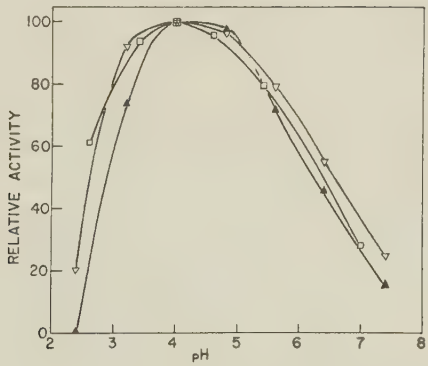

FIG. 1. pH-activity curves for the hydrolysis of α- and β-amylase limit dextrins and of isomaltose by *Candida* amylase. (▽), α-amylase limit dextrin of glutinous rice starch, 0.25% final concentrrtion; (▲), β-amylase limit dextrin of Lintnerized glutinous rice starch, 0.5% final concentration; and (□), isomaltose, 0.002 *M* final concentration.

Fig. 1 shows the relative activity of the enzyme at different pH values, with the per cent substrate hydrolyzed at the optimal pH taken as 100 per cent. It is evident that this optimal is pH 4.0 for the hydrolysis of the three substrates, as has already been reported (*1*) for the hydrolysis of starch, maltose and hetero α-glucosides, and for transfer action upon α-phenyl glucoside. This fact suggests that a single enzyme may be operating upon all

these substrates.

For obtaining information on activity *vs.* enzyme concentration, mixtures were prepared as above, but with buffer set at pH 4.0 and each substrate tested with amounts of enzyme ranging from 25 to 500 units per ml. of

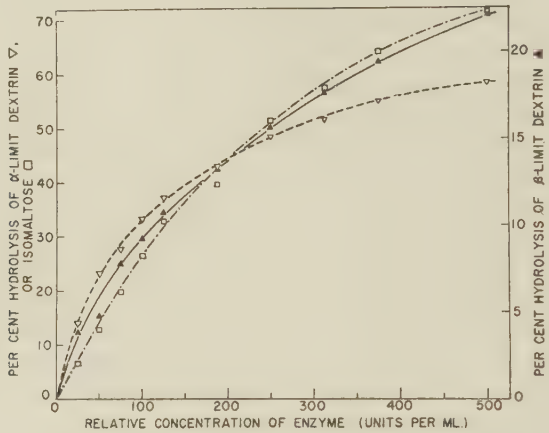

FIG. 2. Relation between enzyme concentration and % hydrolysis of α-amylase limit dextrin (▽); of β-amylase limit dextrin (▲); and of isomaltose (□). See text for reaction conditions.

enzyme solution. Fig. 2 shows the per cent hydrolysis of isomaltose and of α- and β-amylase limit dextrins plotted as functions of enzyme concentration. These curves may be used as standards in estimating the potency of a given enzyme solution from the per cent hydrolysis of any of the three substrates. For this use, as in the case of the following experiment, the substrate concentration, time of incubation, and other conditions must of course be identical with those of the experiments of Fig. 2.

Attempt to Separate the Various Activities of Candida Enzyme Preparations by Zoze Electrophoresis—In an earlier experiment (*1*) a *Candida* enzyme preparation was subjected to continuous paper electrophoresis, using barbiturate buffer of pH 8.1. There was no separation among the capacities to hydrolyze soluble starch, maltose and phenyl α-glucoside or in the capacity to cause glucosyl transfer from phenyl α-glucoside to glycerol. Nevertheless, it seemed worthwhile to extend the attempt at electrophoretic separation of the several activities of *Candida* preparations, by experiments involving different sets of conditions.

Zone electrophoresis, with potato starch grains as the supporting medium, was selected as the basic technique (*8*). Compared with paper electrophoresis, it permitted longer exposure of the enzyme preparation to the electric field as well as the use of low pH values where adsorption might occur in the case of paper (*7*, *8*).

A number of experiments were carried out, using different buffer systems (phthalate-HCl, borate-succinate, borate-phosphate) and different pH

values (ranging from 2.8 to 7.4). All gave results similar to those of the following experiments.

Electrophoresis at pH 7.0 Quality grade raw potato starch, suspended in 0.05 ionic strength borate-phosphate buffer (pH 7.0), was packed into a 45×7 ×1 cm. horizontal plastic trough. After removal of excess buffer, a mixture of starch grains wet by the addition of *ca.* 2 ml. of crude *Candida* enzyme solution (*1*) was introduced into a 1 cm. slit cut across the starch block approximately 6 cm from the cathode. A constant potential of 350 volts (average, 35 mA) was applied for 29 hours at a temperature of approximately 10°–12°. The starch block then was cut crosswise into 1 cm. sections, each of which was extracted with 7 ml. of dilute (0.01 *M*) McIlvaine buffer, pH 4.0.

In one experiment, the capacities to hydrolyze maltose and Yashima Co. soluble starch as well as to transfer the glucosyl group from α-phenyl glucoside to glycerol were determined for each fraction according to the procedures previously reported (*1*). In a second experiment, made under essentially the same conditions, the capacities to hydrolyze soluble starch, α- and β-amylase limit dextrins, and isomaltose were examined. The latter three activities were assayed by the procedures of the experiment of Fig. 2.

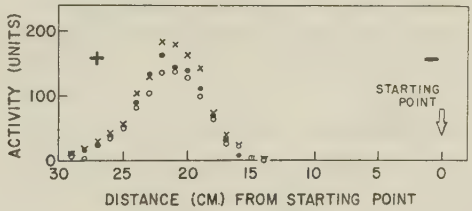

FIG. 3. Electrophoresis of *Candida* enzyme preparation in pH 7.0 phosphate-borate buffer. Activity (units) expresses capacity of individual fractions to hydrolyze soluble starch (●); to hydrolyze maltose (○); and to transfer glucosyl unit from α-phenyl glucoside to glycerol (×).

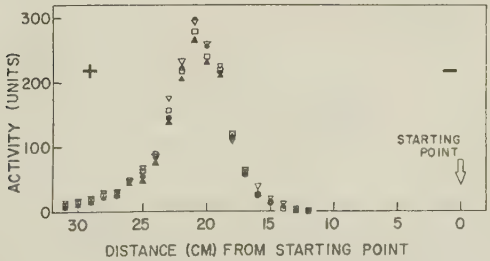

FIG. 4. Electrophoresis of *Candida* enzyme preparation in pH 7.0 phosphate-borate buffer. Activity (units) expresses capacity of individual fractions to cause hydrolysis of soluble starch (●); α-amylase limit dextrin (▽); β-amylase limit dextrin (▲); and isomaltose (□).

As evident from Figs. 3 and 4, all six activities showed the same electrophoretic distribution. Fractions with maximal activity were found, in each case, to have migrated 21 to 22 cm. toward the anode. The electrophoretic migration toward the anode doubtless was actually much greater, since there was considerable endosmotic flow in the opposite direction.

Electrophoresis at pH 2.8 An experiment, comparable to the above, was made using 0.05 ionic strength phthalate-HCl buffer, pH 2.8. The applied potential was 300 volts (average 30 mA) for 30 hours, and each 1 cm. section was assayed for all six activities. As shown in Fig. 5, the distribution

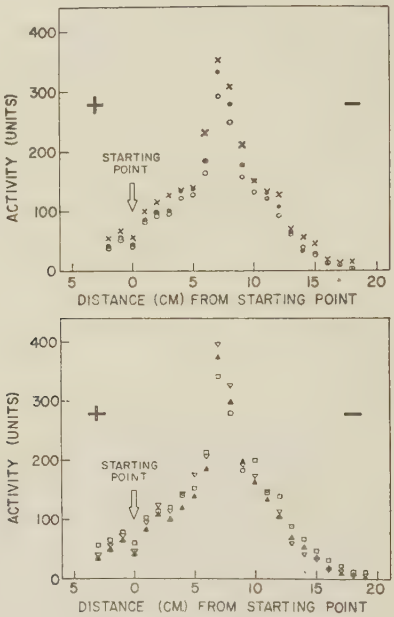

FIG. 5. Electrophoresis of *Candida* enzyme preparation in pH 2.8 phthalate-HCl buffer. Symbols as in Fig. 3 and 4.

curves for all enzymic activities were not significantly different from one another, either in shape or in position of the peak. Maximal activities were associated with a fraction that had migrated approximately 7 cm. toward the cathode. In this case, about 20 ml. of buffer was observed to have transferred from the anode to the cathode reservoir, which extent of electroendosmosis would account for at least 10 cm. of movement to the cathode of the liquid phase in the starch block. Hence, it is likely that the real direction of electrophoretic migration of the enzyme was toward the anode. Accordingly, the isoelectric point of the *Candida* amylase is probably near 2.8, and may actually lie below the value. At any rate, these results of electrophoresis strongly support the concept that the *Candida* amylase itself has all the activities mentioned above.

Influence of Various Carbohydrates upon the Utilization of Soluble Starch and of α-Phenyl Glucoside by the Candida Amylase—If, as appears to be the case, the *Candida* amylase is able to split heterosaccharides, *e.g.*, α-phenyl glucoside, as well as starch, this enzyme would be set apart from all other known saccharogenic amylases, including mold amyloglucosidases. In order to obtain further information bearing on this point, a comparison was made of the inhibitory effects of various carbohydrates on the capacities of a resin-treated *Candida* enzyme preparation to split soluble starch and α-phenyl glucoside.

Each test mixture (T) comprised: 1 per cent Yashima Co. soluble starch, or 0.02 *M* α-phenyl glucoside, in 0.1 *M* McIlvaine buffer at pH 4.0, 2 ml.; enzyme solution containing 200 units, 1 ml.; and carbohydrate solution used as inhibitor, 1 ml. Final concentration of inhibitor was 0.05 *M* except in the case of soluble starch and β-amylase limit dextrin, where the final concentration was 2.5 per cent. Control mixtures of substrate plus enzyme (A), or of buffered substrate alone (B), were included in both the starch and α-phenyl glucoside experiments. Under these conditions, the starch-enzyme and α-phenyl glucoside-enzyme control mixtures (A) showed, respectively, 30 per cent and 26 per cent substrate utilization.

After 20 hours' incubation at 37°, 10 ml. of 1 per cent sodium carbonate solution was added to each mixture. In the case of mixtures with starch, 1 ml. of the alkalinized digest was treated with 35 ml. water, 2 ml. of 0.5 *N* HCl, and 2 ml. of 0.008 *M* I_2 in 0.08 *M* KI; the starch content was estimated by measuring the absorbancy at 540 mμ. Per cent inhibition was calculated as $\frac{T_{abs.} - A_{abs.}}{B_{abs.} - A_{abs.}} \times 100$. For the mixture with α-phenyl glucoside, 2 ml. of the alkalinized digest was treated with 13 ml. of 0.1 *N* NaOH and the free phenol estimated by measuring the absorbancy at 287 mμ. Per cent inhibition was calculated as $\frac{A_{abs.} - T_{abs.}}{A_{abs.}} \times 100$.

As illustrated in Fig. 6, there is reasonably close parallelism between the capacity of each of the inhibitory substances to suppress both utilization of starch and α-phenyl glucoside by the *Candida* enzyme preparation. The parallelism again gives strong support to the idea that a single enzyme in the *Candida* extract is responsible for the utilization both of soluble starch and α-phenyl glucoside. The almost complete arrest of both actions by maltose, in particular, appears difficult to interpret in any other way.

Affinity of Substrates and of Inhibitory Substances for the Candida Amylase—Michaelis constants were determined for the major substrates of the *Candida* amylase, *i.e.*, for soluble starch, maltose, α-phenyl and α-methyl glucosides. All experiments were carried out at 37° using resin treated enzyme solution (*1*). Enzyme concentration and time were adjusted, insofar as possible, to enable measurement of the initial velocity. Increase of reducing power was used for estimating reaction velocity with all substrates except α-phenyl glucoside, where the release of phenol was used. K_m values were obtained from graphs plotted according to the procedure of Hofstee

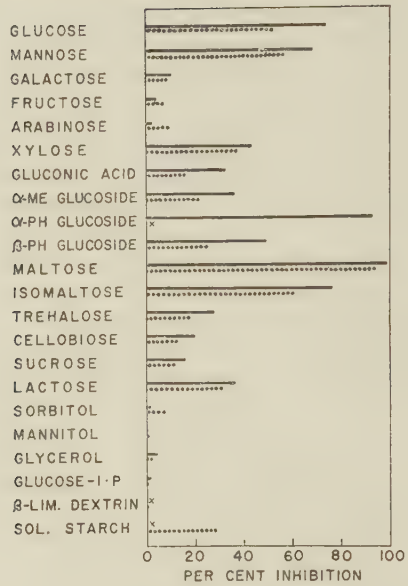

FIG. 6. Inhibition, by various carbohydrates, of the capacity of *Candida* amylase to utilize 0.5% soluble starch (——) or 0.01 *M* α-phenyl glucoside (····). Concentration of inhibitor carbohydrates was 0.05 *M*, except in the case of the dextrin and the soluble starch, which were 2.5%. (×) not tested.

TABLE I

Michaelis Constants of Substrates for Candida Amylase, and Dissociation Constants for Inhibitors of Starch Hydrolysis by the Enzyme

	Michaelis constant, *Km* ($10^{-3}M$)	Second Michaelis constant, *Km'* ($10^{-3}M$)	Dissociation constant, *Ki*, regarding starch hydrolysis ($10^{-3}M$)	Type of inhibition upon starch hydrolysis
Substrate or product				
Soluble starch	0.71(%)[1]	3–4(%)[1]	—	—
Maltose	0.2–0.3	3–4	0.5	Competitive
α-phenyl glucoside	0.5	—	1.4	Competitive
α-Methyl glucoside	16	800–1800	20	Nearly competitive
Glucose	—	—	2.1	Competitive
Non-substrate				
β-phenyl glucoside	—	—	17	Non-competitive
Sucrose	—	—	43	Non-competitive

1) Constants for soluble starch are expressed in per cent instead of *M*.

(*10*). In addition, where inhibition by excess substrate was obtained, K_m' values were calculated (*11*) according to the equation, $K_m' = [S]^2/K_m$, where [S] is the concentration of substrate at which reaction velocity is apparently maximal. The value of [S] was taken from the graphs of reaction velocity *vs.* the logarithm of substrate concentration.

A number of the sugars and glucosides that had been found to inhibit the hydrolysis of soluble starch (Fig. 6) were characterized, at least approximately, by K_i values and "type" of inhibition according to the procedure of Lineweaver and Burk (*12*). Increase of reducing power again was used in estimating reaction velocity, except in the case of inhibition by maltose, where the starch-iodine color reaction was used.

The data summarized in Table I shows that the Michaelis constants, K_m, of maltose, α-phenyl and α-methyl glucosides are of the same order, respectively, as the dissociation constants, K_i expressing their inhibitory action on the hydrolysis of soluble starch. In agreement with the experiment of Fig. 6, increasing K_i values characterized the series: maltose, α-phenyl glucoside, glucose, β-phenyl glucoside, α-methyl glucoside, and sucrose. The type of inhibition of the hydrolysis of soluble starch is competitive in the case of maltose, α-phenyl and α-methyl glucoside, and glucose; whereas, it is non-competitive in the case of the non-substrate carbohydrates, sucrose

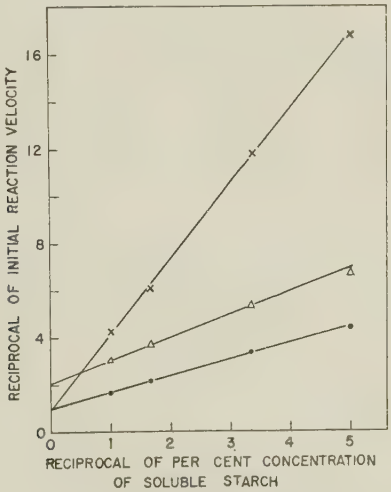

FIG. 7. Inhibitory effects of 0.005*M* α-phenyl glucoside, (×), and of 0.01*M* β-phenyl glucoside, (△), upon the hydrolysis of soluble starch by *Candida* amylase. (●) shows hydrolysis without inhibitor.

and β-phenyl glucoside. The striking difference in action of the two isomeric phenyl glucosides is shown in the Lineweaver-Burk plot of Fig. 7.

These data add evidence for the idea that soluble starch, maltose, α-

phenyl glucoside and α-methyl glucoside are substrates of one enzyme. It is rather unusual that hetero glucosides and starch should be split by the same enzyme, though this has been claimed for an enzyme in equine serum (*14*). The capacity of the *Candida* amylase to do so is strongly indicated by the present experiment as well as by the other experiments of the present paper.

DISCUSSION

The present work has extended previous findings that an intracellular amylase of a wild yeast, *Candida tropicalis* var. *japonica*, has an unusually broad specificity. That is, this enzyme can hydrolyze not only amylosaccharides (soluble starch, α- and β-amylase limit dextrins) but also certain oligosaccharides (maltose and isomaltose) and certain heterosaccharides (α-methyl and α-phenyl glucosides). Glucose is the main sugar produced in these hydrolyses, but the enzyme also can transfer the glucosyl residue to acceptors other than water, *e.g.*, to glycerol. α-Phenyl glucoside was used in the present work as donor to illustrate the transfer potentiality, but starch and other substrates can be donors for the glucosyl transfer, as reported tentatively in 1957 (*13*).

Enzymes capable of releasing glucose from amylopolysaccharides and maltose, and in other ways approaching the broad specificity of the *Candida* enzyme, have been reported in the serum of cattle and horses. The specificity of the equine enzyme extends to the hydrolysis of isomaltose, turanose and α-phenyl glucoside but, in contrast to the *Candida* enzyme, no evidence has been presented that it has transglycosylase activity (*14*). The bovine enzyme has been found to form α-1,4-linked oligosaccharides from maltose by transferring action, but it does not act upon isomaltose or α-methyl glucoside (*15*, *16*, *17*). The *Candida* enzyme thus appears rather unique in its wide range of activities even when compared with these serum enzymes which themselves have very broad substrate specificity.

SUMMARY

The action of amylase preparations of *Candida tropicalis* var. *japonica* was shown to extend to the hydrolysis of isomaltose and α- and β-amylase limit dextrins, with the same optimal pH (4.0) as previously found for hydrolysis of soluble starch, maltose and hetero α-glucosides, and for transglucosylation from α-phenyl glucoside to glycerol. Starch block electrophoresis of several enzyme preparations, using buffers of widely separated pH (7.0 and 2.8), showed that all of the above activities had the same electrophoretic distribution, strongly suggesting that a single enzyme mediates all activities.

Additional evidence on the same point was obtained through studies involving the inhibitory action of various carbohydrates on starch hydrolysis by *Candida* amylase preparations. Thus, a remarkable parallelism was found between the capacity of certain carbohydrates to suppress the utilization of

soluble starch and their capacity to suppress the splitting of α-phenyl glucoside. Maltose, in particular, was found to be an extremely potent inhibitor of both activities. Moreover, the Michaelis constants for maltose, α-methyl glucoside and α-phenyl glucoside were found to be of the same order, respectively, as the dissociation constants obtained when these substrates were used to inhibit the hydrolysis of soluble starch. Finally, the inhibition of starch hydrolysis by these three compounds, as in the case of glucose, proved to be competitive.

The author wishes to express his thanks to Prof. T. Miwa and to Prof. T. Mori for their valuable suggestions and encouragement; to Dr. K. Shibasaki for a supply of isomaltose and to Dr. N. Suzuki for making available the apparatus for electrophoresis. Prof. Edward J. Hehre of the Albert Einstein College of Medicine, New York, helped prepare the manuscript for which the author is very grateful.

REFERENCES

(*1*) Sawai, T., *J. Biochem.*, **45**, 49 (1958)
(*2*) Phillips, L. L., and Caldwell, M. L., *J. Am. Chem. Soc.*, **73**, 3563 (1951)
(*3*) Fukumoto, J., and Tsujisaka, Y., *Science and Industry* (Japan), **30**, 398 (1956)
(*4*) Okazaki, H., *J. Agr. Chem. Soc. Japan*, **32**, 316 (1958)
(*5*) Fukumoto, J., and Tsujisaka, Y., *Science and Industry* (Japan), **32**, 374 (1958)
(*6*) Somogyi, M., *J. Biol. Chem.*, **195**, 19 (1952)
(*7*) Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, **35**, 89 (1952)
(*8*) Kunkel, H. G., and Slater, R. J., *Proc. Soc. Exptl. Biol. Med.*, **80**, 42 (1952)
(*9*) Meyer, K. H., Spahr. P. F., and Fischer, E. H., *Helv. Chim. Acta*, **36**, 1924 (1953)
(*10*) Hofstee, B. H. J., *Science*, **116**, 329 (1952)
(*11*) Friedenwald, J. S., and Maengwyn-Davies, G. D., "A Symposium on the Mechanism of Enzyme Action," ed. by McElroy, W. D., and Glass, B., Baltimore, p. 180 (1954)
(*12*) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934)
(*13*) Sawai, T., "Abstracts of Preliminary Meeting for International Symposium on Enzyme Chemistry," Tokyo, p. 123 (1957)
(*14*) Lieberman, I., and Eto, W. H., *J. Biol. Chem.*, **225**, 899 (1957)
(*15*) Miller, K. D., and Copeland, W. H., *Biochim. et Biophys. Acta*, **22**, 193 (1956)
(*16*) Miller, K. D., *J. Biol. Chem.*, **231**, 987 (1958)
(*17*) Miller, K. D., and Copeland, W. H., *J. Biol. Chem.*, **231**, 997 (1958)

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A SIMPLIFIED CHROMATOGRAPHIC PURIFICATION OF CORD FACTOR

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A toxic lipide component of the tubercle bacillus, which was termed "cord factor", was firstly described by Bloch (*1*). This bacterial constituent is characterized by its occurrence in strains of *Mycobacterium tuberculosis* and by a particular delayed toxicity for mice upon repeated injections of small amounts. Cord factor was obtained as a highly purified preparation from wax fraction (*2*). Chemical structure of cord factor was shown to be trehalose-6,6′-dimycolate by the chemical analysis and synthesis (*3*, *4*).

Cord factor was purified chiefly by the use of column chromatography of magnesium silicate from Wax C by Noll and Bloch (*5*), while Nojima (*6*) fractionated Wax D by magnesium silicate chromatography and proposed that he could obtain cord factor in a good yield. The present paper describes a simplified method for obtaining highly purified preparation of cord factor from the lipide component of proteolipide, which was prepared according to the method of Yamamura (*7*).

EXPERIMENTAL AND RESULTS

Isolation of Lipide Component of Proteolipide—The tubercle bacilli, BCG strain, were grown in surface culture on Sauton's media for 4 weeks. The organisms were autoclaved at 120° for 20 minutes, collected by filtration, washed thoroughly with distilled water and collected on the filter paper.

An adaptation of Yamamura's method was employed with some minor modifications. Five hundred grams of heat killed wet bacilli were homogenized with 5 liters of the mixture of chloroform and methanol (v/v 2 : 1) and extracted for 2 days in an ice box. At the end of this period, the extract was obtained by the filtration through a glass filter and celite. The extract was concentrated to about 500 ml., then added with 2.5 liters of acetone-methanol (1 : 1) and allowed to stand for several hours in an ice box. The precipitate was collected by filtration, dissolved in a small amount of chloroform-methanol (2 : 1) and precipitated again with five volumes of acetone-methanol (1 : 1). This proucedure was repeated for several times

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and the final solution in chloroform-methanol was heated at 80° for one hour to split off the linkage and protein moiety of proteolipide in the solution. Five volumes of acetone-methanol (1 : 1) was added to the heated solution. Precipitates were collected by filtration and dissolved in benzene, and the insoluble part was removed by centrifugation at 19,000 r.p.m. for 40 minutes. Acetone-methanol (1 : 1) was added to the supernatant benzene solution and precipitates were collected by filtration. The dissolution and precipitation procedures were repeated for several times.

The white waxy substance termed PL fraction, is a major part of lipide component of the cavity inducing proteolipide, as has been reported by Yamamura (*7*). The yield is around 5 g. from 500 g. of wet cells.

Chromatographic Purification of Cord Factor from PL Fraction—For the chromatographic procedures, silica gel (Kanto Kagaku Co. Ltd.) was used as adsorbents. The result of the first column chromatography is shown in Table I. One gram of PL was dissolved in petroleum ether and adsorbed on 40 g. of silica gel and 20 g. of Hyflo-supercel. PL fraction was separated into eight fractions as shown in Table I.

TABLE I

Chromatogram I. Chromatography of Crude Cord Factor on Silica Gel

One gram of material was adsorbed on 40 g. of silica gel and 20 g. of Hyflo-supercel.

Fraction No.	Eluted with 700 ml. of		Sample eluted	M. P.
			mg.	°C
1	petroleum ether		8	
2	Petroleum ether-ether	19 : 1	289	46 – 47
3	,, ,,	9 : 1	80	46.5 – 48
4	,, ,,	4 : 1	98	53 – 55
5	,, ,,	1 : 1	260	44
6	,, ,,	1 : 4	45	
7	Chloroform methanol	4 : 1	128	54.5 – 56.5
8	Methanol		60	187 – 188
	Recovered		968 mg. = 96.8%	

The chloroform-methanol eluate fraction (Fraction No. 7) was rechromatographed on silica gel as shown in Table II. One hundred and twenty-eight mg. of Fraction 7 in Table I dissolved in chloroform were adsorbed on the mixed column of 5.6 g. of silica gel and 2.8 g. of Hyflo-supercel and separated into 6 fractions by the elution as shown in Table II.

Toxicity Test—Toxicity of the four rechromatographed fractions (c, d, e,

TABLE II

Chromatogram II. Chromatography of Chloroform-Methanol Fraction Obtained after Chromatography I on Silica Gel

One hundred and twenty-eight mg. of material were adsorbed on 5.6 g. of silica gel and 2.8 g. of Hyflo-supercel.

Fraction No.	Eluted with 100 ml. of			Sample eluted	M. P.
				mg.	*°C*
a	chloroform			66	
b	chloroform-methanol		99 : 1	1	43 – 46°
c	,,	,,	98 : 2	25	41 – 43°
d	,,	,,	95 : 5	38	42 – 43°
e	,,	,,	90 : 10	22	51 – 70°
f	,,	,,	80 : 20	11	190 – 200°
Recovered				103 mg. = 80.5 %	

TABLE III

Toxicity of Each Fraction Isolated by Chromatography on Silica Gel vacuo

No. of mice tested	Fraction (Table II)	Body weight decrease of mice[1)]	Survival time (days)	Number of survivors (after days)
10	c	+	3,3,4,5,5,5,6	1
10	d	++	3,3,3,3,3,3,3, 3,4,5	0
10	e	—		10
10	f	—		10
10	control (paraffin oil)	—		10

1) Ten μg. each fraction isolated by the second chromatography on silica gel were injected intraperitoneally. Mice, dd strain, body weight 10–15 g., male.

The mark + indicates 10 % or less decrease, ++ indicates 10–20 % decrease and — indicates no decrease of body weight of mice.

f Fraction of Table II) was tested on mice, dd strain, body weight 10–15 g., male. The injection was made intraperitoneally once per two days in a dosis of 10 μg. of each fraction dissolved in 0.1 ml. of paraffin oil. The result is shown in Table III, indicating that the d fraction is the most toxic and seemed to be cord factor.

Elementary Analysis and Infra-red Spectrum—Elementary analysis of a fraction showed the following data;

	C	H
Found	77.75	12.90
Calcd. for $C_{186}H_{366}H_{17}$ (cord factor):	77.70	12.83

The spectrum of d fraction (Fig. 1, Curve 1) shows well defined bands all associated with cord factor. The exact positions of each adsorption

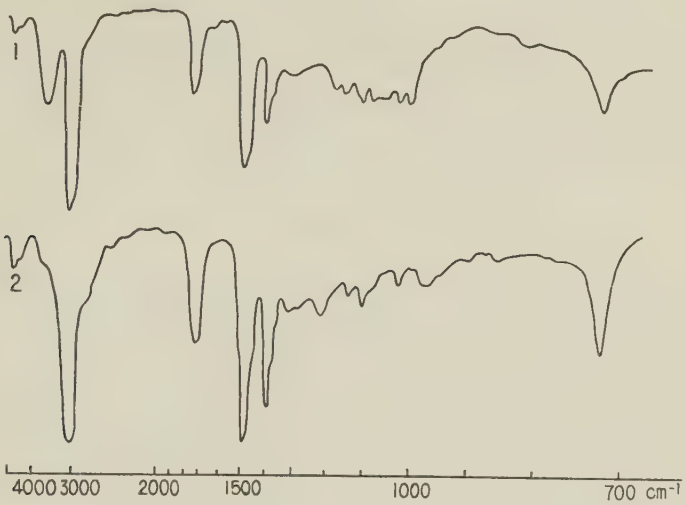

Fig. 1. Infrared spectra Curve 1. Fraction d in Chromatorgraphy II. Curve 2. Ether extratct of acid hydrolysate of Fraction d.

maximum are 3350, 2900, 2840, 1715, 1470, 1380, 1275, 1170, 1156, 1100, 1075, 1050, 1020, 995, 915, 805 and 720 cm^{-1}.

Chemical Degradation—The toxic compound, d fraction, was hydrolysed according to the method of Noll and Bloch (*5*). Two hundred and eighty-three mg. of the d fraction were suspended in a solution of 5 ml. of isopropanol, 0.1 ml. of water, and 100 mg. of KOH. The solution was refluxed for one hour, cooled, acidified with 2 ml. of *N* HCl and extracted repeatedly with an excess of petroleum ether and ether. The extracts were taken to dryness and the lipide residue was dissolved in a small volume of ether and precipitated with methanol. The precipitate was collected and dried *in vacuo* at room temperature; 231.8 mg. of a white powder, m.p. 55–57° were obtained. Found, C 81.48, H 13.38. These values agree with those by Aebi *et al.* for α-mycolic acid Brevannes $C_{87}H_{174}O_4 \pm 5CH_2$ (*8*). The infra-red spectrum (Fig. 1, Curve 2) is identical with that of α-mycolic acid Brevannes.

The acidified water solution obtained after alkaline hydrolysis was deionized by passing through a mixed anion-cation exchange resin (IR-120 (H^+) and IR-4B (OH^-)) and concentrated *in vacuo.*

The concentrate was paperchromatographed on Toyo filterpaper No. 51 with 80 per cent phenol and *n*-butanol-ethanol-ammonia-water (40 : 10 : 1 : 49) as solvents in ascending. Spot was detected at the R_f of 0.30 in the case of former solvent system, and 0.04 in the case of latter solvent system.

These R_f values were in accordance with those of authentic trehalose.

The concentrate was dissolved in 3 *N* HCl, and hydrolysed for 4 hours at 100°, after passing through Amberlite IRA 400 (OH^-). The hydrolysate was concentrated under reduced pressure. Glucose in the concentrate was identified by paperchromatography, since R_f was 0.37 by 80 per cent phenol and 0.14 by *n*-butanol-ethanol-ammonia-water (40 : 10 : 1 : 49) as solvent system.

From these findings it became clear that the toxic d fraction, which was isolated by our method, was an mycolic acid ester of trehalose and identical with cord factor presented by Bloch *et al.* (*1–3*).

DISCUSSION

Chromatographic isolation of cord factor using silicic acid has been successfully reported by Noll and Bloch (*5*) and Nojima (*6*). In these reports Wax C or D fraction from Purified Wax was materials for the isolation of cord factor. In the present study lipide component (PL fraction) of proteolipide, a cavity-inducing substance, was chromatographed using silica gel and cord factor was easily and reproducibly obtained. It was found by Nagasuga *et al.* (*9*) that cord factor could be obtained by the same chromatographic method from Wax C, Wax D and purified Wax. From these results, it is assumed that this chromatographic method on silica gel will be used as a simplified method for the isolation of cord factor.

SUMMARY

Lipide component of proteolipide was fractionated by silica gel chromatography and cord factor was isolated in a good yield.

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REFERENCES

(*1*) Bloch H., *J. Exptl. Med.*, **91**, 197 (1950)
(*2*) Noll, H., and Bloch, H., *Amer. Rev. Tuberc.*, **67**, 828 (1953)
(*3*) Noll, H., and Bloch, H., *Biochim. et Biophys. Acta*, **20**, 299 (1956)
(*4*) Gendre. T., and Lederer, E., *Bull. Soc. Chim.*, 1478 (1956)
(*5*) Noll, H., and Bloch, H., *J. Biol. Chem.*, **214**, 251 (1955)
(*6*) Nojima, S., *J. Biochem.*, **46**, 499 (1959)
(*7*) Yamamur, Y., *Adv. Tuberculosis Research*, **9**, 13 (1958)
(*8*) Aebi, A, Asselineau, J., and Lederer, E., *Bull. soc. chim. biol.*, **35**, 661 (1953)
(*9*) Nagasuga, T., Terai, T., and Yamamura, Y., *Amer. Rev. Resp. Dis.*, in press.

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STUDIES ON BACTERIAL AMYLASE

IV. ON THE C-TERMINAL REGION OF BACTERIAL AMYLASE

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N-Terminal group (*1*) and the terminal pentapeptide sequence (*2*) of bacterial α-amylase (BA) produced by *Bacillus subtilis* N′ were elucidated in previous studies of this series. It was demonstrated that the N-terminal amino acid of the enzyme differed from that of Taka-amylase A (*3*) but the terminal peptide sequence showed a significant similarity to that of Taka-amylase A (*4*, *5*). Ikenaka (*6*) demonstrated that each one mole of glycine, alanine and serine occupied C-terminal positions of Taka-amylase A, and postulated a branched peptide structure with one N-terminal alanine for the amylase. Elucidation of C-terminal group of bacterial amylase was therefore attempted for the purpose of comparing the structnre of both enzymes. Yoshida (*7*) has recently reported that C-terminal group of a bacterial amylase, produced by a different strain (*Bacillus subtilis* K), is asparagine and that N-terminal peptide seemed to be the same as that of the amylase from strain N′.

C-Terminal analysis of the strain N′ enzyme was performed by using hydrazinolysis method (*8*) and carboxypeptidase A (*9*) in the present study. The results obtained by both methods did not agree with each other. Moreover it was unsuccessful to indicate that asparagine located at C-terminal end of the enzyme. The discrepancy between the result of Yoshida and that of the present author might be due to the difference of bacterial strains used by the two workers. Although an ambiguity is still left on the C-terminal group, at least three amino acid residues near C-terminus appear to be not essential for α-amylase activity.

MATERIALS AND METHODS

Bacterial Amylase—The crystalline BA was kindly supplied by Nagase Co. Ltd. In some cases this enzyme preparation was further recrystallized twice by the method of Hagihara (*10*).

Anhydrous Hydrazine—This reagent was prepared according to the method of Stähler (*11*). The analysis showed that it was over 98 per cent purity.

Carboxypeptidase (A)—Diisopropylphosphofluoridate treated carboxypeptidase was obtained from Worthington Biochemical Co., Freehold, New Jersey, and was used, as water suspension.

Silica Gel—This was prepared by a similar method as described previously (*12*).

Solvent systems employed on the silica gel column chromatography are as follow.

A : Butanol : chloroform : acetic acid : water (1 : 4 : 1 : 4)

B : Chloroform : acetic acid : water (5 : 1 : 4)

Hydrazinolysis, Dinitrophenylation, Aldehyde Treatment and Extraction—One hundred milligrams of BA were hydrazinolysed with about 1 ml. of anhydrous hydrazine at 100°, the time of the reaction varying from three to fifteen hours. Excess hydrazine was removed *in vacuo* over sulfuric acid. The dinitrophenylation and benzaldehyde or isovaleraldehyde treatment of the hydrazinolysate, and the fractional extraction of the C-terminal DNP-amino acid from di- and tri-DNP-derivatives of hydrazides were essentially the same as the standard method I recommended by Akabori and coworkers (*8*).

Identification of C-Terminal Amino Acid by Dowex-50 Column Chromatography—The aqueous phase obtained by the benzaldehyde treatment of the hydrasinolysate of BA was evaporated in a vacuum desiccator. The residue was dissolved in an appropriate amount of buffer (pH 3.42) and placed on the top of the ion exchange resin column. The amount of the hydrazinolysate applied corresponded to about one μmole of BA in the case of 0.9×100 cm column and to about 0.6 μmole of BA in the case of 0.9×15 cm column. The buffer solutions employed in this experiment and most part of the procedure were the same as reported by Moore and Stein (*13*).

Carboxypeptidase Digestion—This experiment was carried out by Harris' method (*9*). About 2–3 μmoles of lyophilized or crystalline BA was dissolved in water and pH was adjusted to 8 with dilute NaOH solution (protein concentration was 0.1–1.0 per cent). The protein solution was digested at pH 8–8,5 and at room temperature or 37° with various ratios of the substrate to the enzyme (30, 50, 60 and 100 to 1). An aliquot containing approximately 0.2–0.4 μmoles of BA was removed at various time intervals and each aliquot was immediately applied to the Dowex-50 ($\times 2$, H^+ form) column (0.9×5.0 cm) or analyzed directly by the DNP method (*14*). In the former case, after the column was washed with deionized water to remove protein, the amino acids were eluted from the resin with 5 *N* aqueous ammonia. The eluates were evaporated to dryness *in vacuo* over concentrated H_2SO_4 and were subjected to paper chromatography with butanol-acetic acid-water (4 : 1 : 2) or 80 per cent phenol.

Chromatographic Identification of C-Terminal Amino Acid—DNP-Derivatives of C-terminal fraction were fractionated and identified by silica gel column chromatogrphy or two dimensional paper chromatography with solvent system of Levy (*15*) or Koch (*16*). Each band of DNP-amino acid on the column chromatogram was cut off, extracted with ethylacetate or methylalcohol, and dried *in vacuo*. The residue was disssolved in 5 ml. of 1 per cent sodium bicarbonate solution and determined colorimetrically at 430 mμ.

Each spot on the paper chromatogram was cut out and extracted with 4 ml. or 5 ml. of 1 per cent sodium bicarbonate solution in a water-bath at 55–60° for 15 minutes or at room temperature for about two hours. The solution was filtered in the former case or decanted in the latter case and the optical density was read at 360 mμ with Shimazu photoelectric spectrophotometer.

α-Amylase Activity—Amylase activity was measured at pH 5.6 (*M*/2 Acetate buffer) and 30° for 10 minutes, using 0.1 per cent amylose solution as substrate, otherwise Fuwa's method (*17*) was followed.

RESULTS AND DISCUSSION

Investigation of C-Terminal Amino Acid by Hydrazinolysis—A chromatography of DNP-derivative of C-terminal fraction obtained by hydrazinolysis was achieved by means of a silica gel column. The chromatograms showed

that the C-terminal fraction contained mainly glycine and a small amount of aspartic acid, serine, alanine, threonine and valine. In the case where the band on the silica gel column chromatogram was contaminated with DNP-derivatives of acidic amino acid hydrazides, it was subjected to paper electrophoresis for 2 hours, using lutidine-*M*/15 acetic acid buffer of pH 6.5 (5 : 100, vol.) and 12.5 volts/cm, in order to purify the DNP-amino acid in the band.

In addition, DNP-derivatives of the C-terminal fraction obtained by the standard method III of Akabori *et al.* (without aldehyde treatment) was chromatographed on paper, and the chromatogram is given in Fig. 1.

This chromatogram also shows that the main amino acid in the C-terminal fraction is glycine.

The DNP-glycine obtained by silica gel column and paper chromatography was regenerated with saturated barium hydroxide and the free amino acid produced was identified as glycine by paper chromatography with butanol-acetic acid-water (4 : 1 : 5, vol.).

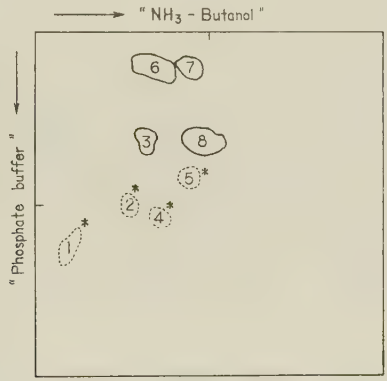

Fig. 1. Paper chromatogram of DNP-derivatives of amino acids released by hydrazinolysis.

1. DNP-Asp+DNP-Glu 2. DNP-Ser 3. DNP-Gly 4. DNP-Thr 5. DNP-Ala 6,7. di-DNP-Acidic Amino Acid monohydrazide 8. Dinitrophenol

* Dotted spot shows a faint color.

To investigate the possibility of the presence of histidine at C-terminus as preliminarily reported by Akabori and Ohno (*18*), and to obtain further information on the main component contained in the C-terminal fraction, the chromatographic analysis of the hydrazinolysate on Dowex 50 column (×8, Na^+ form) was performed.

As is shown in Fig. 2, histidine was not recognized in the C-terminal fraction. The quantitative results on these chromatograms are summarized in Table I.

The results mentioned above showed that free amino acids obtained by hydrazinolysis were aspartic acid, serine, glycine, alanine, threonine and

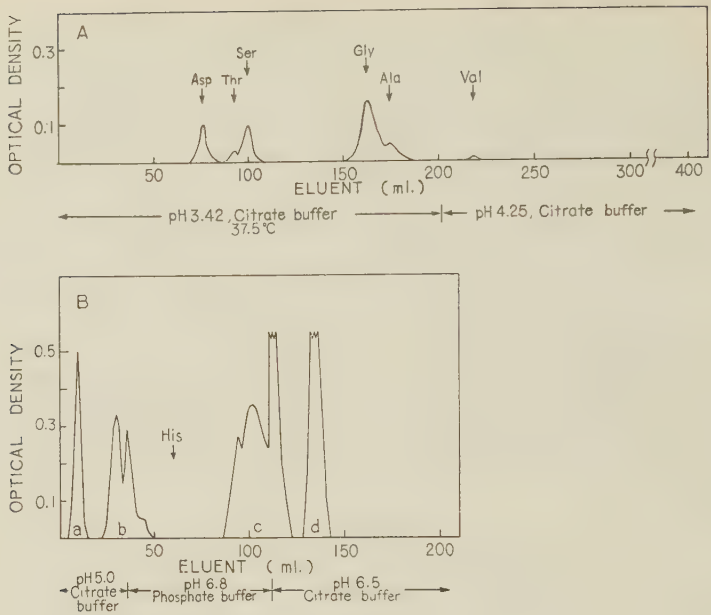

FIG. 2 Elution curve for the 10 hours hydrazinolysate of BA treated with benzaldehyde.

(A) on a 0.9×100 cm. column of Dowex 50×8

(B) on a 0.9×15 cm. column of Dowex 50×8

Peak (a) is acidic and neutral amino acids.

Peaks, (b), (c) and (d), are supposed to be benzaldehyde derivatives of acidic amino acids monohydrazides, or water soluble benzaldehyde derivatives of some amino acid hydrazides.

valine. But amounts of five amino acids except glycine are too small to be accepted as C-terminal group. Bradbury (*20*) has recently reported that small amounts of non-C-terminal amino acids, such as glycine, serine, glutamic and aspartic acid, are produced during hydrazinolysis. Accordingly, it is difficult to assume that the above-mentioned minor components are C-terminal group of BA. Judging from these results, it is probable that the C-terminus of this amylase is glycine. In order to confirm this point carboxypeptidase experiment was then carried out.

Carboxypeptidase Digestion—BA was digested by carboxypeptidase with several ratios of the enzyme to substrate (1:57 and 1:30 at 37°, and 1:85 at room temperature). During digestion, the pH was maintained at 8.0–8.5 by the addition of dilute sodium hydroxide.

Leucine and/or isoleucine, valine and phenylalanine were released at faster rates than other amino acids from BA with carboxypeptidase A as can be seen in Fig. 3. But the order of liberation of above three amino acids differed from experiment to experiment. When alkali denatured BA (crystalline BA was dissolved in 0.1 *N* NaOH and kept in a refrigerator

TABLE I

C-Terminal Amino Acid of BA[1] *by Hydrazinolysis*

Time of hydrazinolysis		Residues per mole protein (molecular weight 48,700) (*19*)						
		Asp	Glu	Ser	Gly	Ala	Thr	Val
3	A			0.15	0.27			
	A			0.23	0.35	0.11	0.16	
5	A	0.24		0.34	0.59	0.29	0.22	
	B		0.26	0.24	0.36		0.26	
10	A	0.16		0.23	0.46	0.12	0.14	0.13
	B		0.19	0.32	0.55	0.27	0.45	
	B		0.17	0.22	0.56	0.37	0.10	
	C	0.06		0.23	0.56	0.11	0.06	0.01
15	A	0.16		0.23	0.46	0.12	0.14	0.13
	B		0.19	0.32	0.55	0.27	0.45	

1) The values obtained by DNP-method are uncorrected for hydrazinolysis.

A: Silica gel column chromatography as DNP-derivatives; These values were corrected for loss by column chromatography.

B: Paper chromatography as DNP-derivatives.

C: Dowex-50 column chromatography as free amino acid.

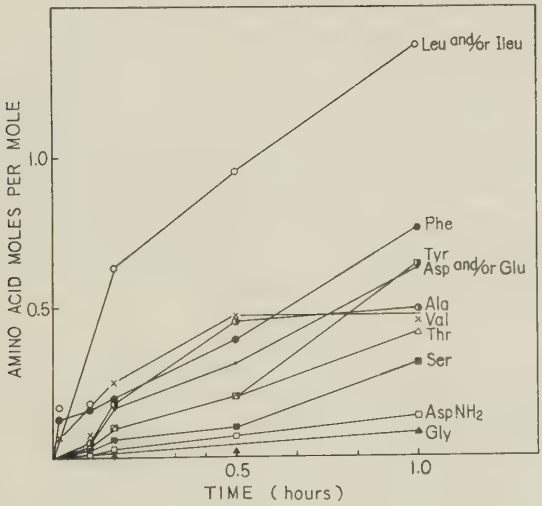

FIG. 3. Amino acids released from BA by carboxypeptidase: BA concentration 0.55%; carboxypeptidase concentration 0.018%; pH 8.5; 24°.

overnight) was incubated with carboxypeptidase, a similar result was obtain-

ed but the reaction rate was faster than with the native one (Fig. 4). If C-terminus of this amylase is either of these three amino acids, (valine, phenylalanine and leucine and/or isoleucine), it must be clearly identified

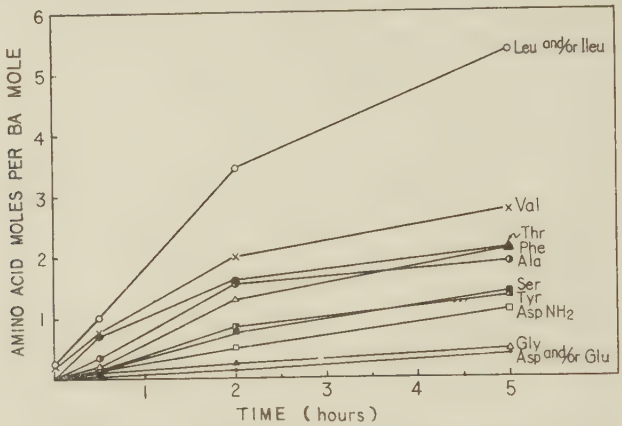

Fig. 4. Amino acids released from denatured BA with alkali by carboxypeptidase: Protein concentration 0.7%; carboxypeptidase concentration 0.014%; pH 8; 25°.

by the hydrazinolysis method, and if the result obtained by hydrazinolysis method is correct, glycine has to be released from BA first, as is shown in carboxypeptidase degradation of horse myoglobin (*21*).

On the other hand, Yoshida reported that C-terminus of α-amylase from *Bacillus subtilis* K was asparagine as described above, and a possibility of C-terminal asparagine in BA (strain N′) was considered. Each aliquot of the incubate with carboxypeptidase for 1, 6 and 10 minutes was analyzed by paper chromatography with a butanol-acetic acid-water (4:1:2) and a 80 per cent phenol prior to dinitrophenylation, and asparagine was not clearly recognized on the chromatograms. But, a small amount of DNP-asparagine was found on the two dimensional paper chromatogram after dinitrophenylation. Therefore, the recovery of asparagine was measured by chromatography after dinitrophenylation. (Table II).

Table II

Recovery of Asparagine by Dinitrophenylation

Incubation time at pH 8 (hours)	0	24	48
Recovery of DNP-asparagine (%)	55	57	38
Recovery as DNP-aspartic acid (%)	2	3	2

Even though the loss of asparagine and the degradation into aspartic acid during dinitrophenylation is taken into consideration, it is hard to assume that asparagine is a real C-terminus of BA of the strain N′. Thus,

TABLE III

Amino Acids Released from BA by Carboxypeptidase and α-Amylase Activity

Condition: protein concentration 0.9%, carboxypeptidase concentration 0.008%, pH 8.5, 22–24°.

Time (hours)	Amino acid released (moles per mole of BA)											Remaining α-amylase activity (per cent)
	Val	Phe	Leu &/or Ileu	Thr	Ala	Ser	Asp &/or Glu	Tyr	Gly	Asp. NH_2	total	
0												100
1	0.12	0.28	0.17	0.09	0.03	0.03	0.03	0.08	t [1]	0.03	0.85	
3.5	0.09	0.18	0.12	0.13	0.04	0.02	0.04	0.02	t [1]	0.06	0.70	98
6.5	0.31	0.30	0.42	0.38	0.17	0.18	0.13	— [2]	0.02	0.11	2.02	102
10	0.62	0.49	0.50	0.57	0.26	0.21	0.17	0.08	0.02	0.17	3.09	
25												101
48	0.72	0.54	0.46	0.60	0.30	0.21	0.12	0.08	0.18	— [2]	3.21	108
72	1.25	0.83	0.82	0.52	0.30	0.19	0.10	0.18	0.15	— [2]	4.34	100

1) "t" means the trace amount of the DNP-amino acid.

2) The spot of the DNP- amino acid was not recognized on paper chromatogram.

no clear conclusion about the C-terminal group of BA can be drawn in the present experiments. It may, however, be presumed that amino acids located near the C-terminal end of BA are—(Gly, $AspNH_2$, Ser, Thr, Tyr, Asp and /or Glu, Ala)·(Val, Phe, Leu and/or Ileu).

Amino Acids in the C-Terminal Region of BA and α-Amylase Activity—α-Amylase activity of BA during carboxypeptidase degradation was measured and the results are shown in Table III.

The digest of 72 hours was dialyzed against $M/200$ calcium acetate for 2 days and the enzyme in the bag could be crystallized as the same crystalline form (needle) as that of the native BA.

The decrease of α-amylase activity by the carboxypeptidase digestion was not observed even after 72 hours as is shown in Table III. Judging from this result it can be assumed that at least three amino acids (leucine, valine and phenylalanine) which appear to be present in the C-terminal region of BA may not directly be concerned with the α-amylase activity. In order to obtain further evidence, C-terminal amino acid of the "core" should be analyzed and compared with the "native" enzyme. At present, although the "core"-like substance was obtained in crystalline state, the chemical and physical properties of this "core" have not yet been investigated. Therefore, the probability that only the contaminated denatured enzyme was digested by carboxypeptidase and that the "core"-like substance obtained might be native BA itself cannot be ruled out. The study on the above mentioned problem is now in progress.

SUMMARY

The C-terminal amino acid of BA (*B. subtilus* N′) was analyzed by hydrazinolysis and by the use of carboxypeptidase. From the results of hydrazinolysis, it was suggested that C-terminus of BA might be glycine. While, a considerable amount of amino acid was released by the action of carboxypeptidase on native bacterial amylase and no decrease of the amylase activity was observed. Amino acids liberated by carboxypeptidase are leucine and/or isoleucine, valine, phenylalanine, alanine, acidic amino acids, tyrosine, threonine, serine, asparagine and glycine.

In contrast to the case of hydrazinolysis, it is remarkable that leucine, valine and phenylalanine are liberated in much larger amounts as compared to that of glycine. No clear cut result was obtained about the nature of C-terminal group of BA. However, it might be suggested that C-terminus of BA is not essential for the enzymatic activity.

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REFERENCES

(*1*) Sugae, K., *J. Biochem.*, **47**, 170 (1960)
(*2*) Sugae, K., and Honda, Y., *J. Biochem.*, **47**, 307 (1960)

(*3*) Akabori, S., and Ikenaka, T., *J. Biochem.*, **42**, 603 (1955)
(*4*) Narita, K., and Akabori, S., *J. Biochem.*, **46**, 91 (1959)
(*5*) Tsugita, A., *J. Biochem.*, **46**, 583 (1959)
(*6*) Ikenaka, T., *J. Biochem.*, **43**, 255 (1956)
(*7*) Yoshida, A., and Tobita, T., *Biochim. et Biophys. Acta*, **37**, 513 (1960)
(*8*) Akabori, S., Ohno, K., Ikenaka, T., Okada, Y., Hanafusa, H., Haruna, I., Tsugita, A., Sugae, K., and Matsushima, T., *Bull. Chem. Soc. Japan*, **29**, 507 (1956)
(*9*) Harris, J. I., " Methods of Biochemical Analysis " Vol. **II**, Interscience Publishers, New York, p. 397, (1955)
(*10*) Hagihara, B., *Ann. Rep. Scient. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954)
(*11*) Stähler, A., *Ber. deut. Chem. Ges.*, **42**, 3018 (1909)
(*12*) Sakurai, S., and Sugae, K., *J. Biochem.*, **39**, 367 (1955)
(*13*) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192**, 663 (1951)
(*14*) Sanger, F., *Biochem. J.*, **39**, 507 (1945)
(*15*) Levy, A. L., *Nature*, **174**, 126 (1954)
(*16*) Koch, G., and Weidel, W., *Z. physiol. Chem.*, **303**, 213 (1956)
(*17*) Fuwa, H., *J. Biochem.*, **41**, 583 (1954)
(*18*) Akabori, S., and Ohno, K., IIe Congrès International de Biochimie, (Paris) p. 47 (1952)
(*19*) Junge, J. M., Steine, E. A., Neurath, H., and Fischer, E. H., *J. Biol. Chem.*, **234**, 556 (1959)
(*20*) Bradbury, J. H., *Biochem. J.*, **68**, 482, (1958)
(*21*) Holleman, J. W., and Biserte, G., *Biochim. et Biophys. Acta*, **33**, 143 (1959)

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OXIDATIVE PHOSPHORYLATION COUPLED WITH DENITRIFICATION IN INTACT CELL SYSTEMS

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(Received for publication, April 4, 1960)

It has long been presumed that the so-called nitrate respiration involves an energy yielding mechanism corresponding to the oxidative phosphorylation in the aerobic respiration (*1*). It is important and desirable, particularly in view of the comparative biochemistry, to study the energy metabolism in bacteria which use nitrate or nitrite as the terminal electron acceptor instead of oxygen. This was first suggested by Takahashi, Taniguchi and Egami (*2*) in their experiments which showed incorporation of P^{32}-labelled orthophosphate into cells of a facultative anaerobe *Escherichia coli* in the process of nitrate reduction under anaerobic conditions. In the sulfate reduction, Ishimoto (*3*) also showed, by a similar procedure, the incorporation of radioactive orthophosphate into a strict anaerobe, *Desulfovibrio*. In the present study the phosphorylation coupled with the denitrification was studied using intact cells of a strain of *Pseudomonas denitrificans*, a heterotrophic facultative anaerobe. Our results, though the experiments were still preliminary, provide a strong evidence for the occurence of oxidative phosphorylation coupled with the process of denitrification in which nitrate or nitrite is converted into gaseous nitrogen in anaerobic atmosphere.

MATERIALS AND METHODS

Microorganism—The denitrifying bacterium, originally isolated from soil by Iwasaki and Mori (*4*), grown anaerobically for 48 hours at 30° in the medium described in their paper, was used.

Resting Cell Suspension—The bacteria harvested were washed 3 times with 0.85 per cent NaCl solution and suspended in distilled water.

Assay Method—The denitrifying activity was measured in the nitrogen atmosphere by the Warburg method at 30°C. Unless otherwise stated, the reaction system contained a resting cell suspension, H-donor (lactate), H-acceptor (sodium nitrite), magnesium chloride, sodium fluoride, inorganic orthophosphate and Tris-buffer. Mixing P^{32}-orthophosphate, the incorporation of radioactive phosphate into labile acid-soluble phosphoric esters was measured according to the following procedure. After a certain run of the denitrifying reaction, the reaction vessels were chilled in ice-water, cells were collected by centrifugation and extracted with ice-cold 0.6 *N* PCA (perchloric acid). Adding Norit to the supernatant, mixture was stirred well and the adsorbant was washed 3 times with non-labelled phosphate solution and subjected to the acid hydrolysis in *N* HCl for 10 minutes. An

aliquot of the supernatant was dried in a porcelain planchet by irradiation with an infra-red lamp and its radioactivity was counted by a conventional Geiger-Müller counter. That all the radioactivity of this supernatant was due to inorganic phosphate produced by the hydrolysis, was confirmed by the isobutanol-benzene extraction method (*6*). Unless otherwise stated, phosphorylating activity was expressed as the difference of radioactivity between the test and control system which had no substrate in its reaction medium.

Analysis on Nucleotides—The distribution of radioactive phosphates among acid-soluble fractions was examined according to the following procedure. After a certain duration of denitrifying reaction, the frozen cells were washed twice with distilled water and extracted with chilled 0.6 *N* PCA, and washed two times with 0.2 *N* PCA. The extract and washings were combined and neutralized to pH 7.0, and the resulted $KClO_4$ was removed by centrifugation under sufficient cooling. This extract was passed through a 0.5 cm × 20cm column of Dowex-1 anion exchange resin pretreated with formate and the adsorbed substances were fractionated by means of the gradient-elution technique which was virtually the same as those described by Hurlbert *et al.* (*7*).

Thus, the chromatographic elution analysis was carried out, passing through a 100 ml. mixing volume, successively with the following eluants, H_2O 50 ml., 1 *N* formic acid 50 ml., 4 *N* formic acid 180 ml., 0.2 *M* ammonium formate in 4 *N* formic acid 150 ml., 1 *M* ammonium formate in 4 *N* formic acid 130 ml. With each fraction, which was about 4 ml. in volume, the optical absorption at 260 mμ and 280 mμ and the radioactivity were measured. The latter was carried out in solutions using a conventional dipcounter of GM-type.

RESULTS AND DISCUSSION

Dependence on Length of Reaction Time—The processes of denitrification and coupled phosphorylation are presented together in Fig. 1. In this experiment lactate was used as H-donor and sodium nitrite as H-acceptor. When the cells were kept in contact with P^{32}-labelled orthophosphate in the medium 15 minutes prior to the starting of the reaction, both denitrifying and phosphorylating reactions showed no lag period. While the denitrifying reaction proceeded linearly with time more than 30 minutes, the labile phosphate reached its maximal concentration within 10 minutes under the given conditions.

Effect of pH—Effect of pH on the denitrifying and phosphorylating activity is shown in Fig. 2. To provide the desired pH, 0.025 *M* (final concentration) Tris-buffer at each pH value was added to the medium. The maximum rate of phosphate esterification occurred at about pH 8.2. Denitrification had its pH optimum at 7.0 or slightly less. In this experiment, 10 minutes' reaction time was chosen in order to compare the initial reaction rates of phosphorylation.

Dependence on the Phosphate Concentration—Under the experimental conditions employed, both phosphorylation and denitrification appreciably depended on the phosphate concentration in the reaction medium as shown in Fig. 3. Both activities did not attain optimum values till the concentration of phosphate was as high as 20 μmoles per 3 ml. The efficiency of phosphate incorporation induced by denitrification, being expressed in terms

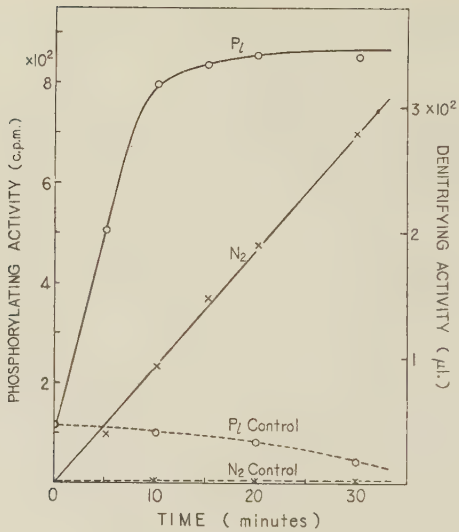


FIG. 1. Time course of denitrifying and phosphorylating activity.
× denitrification, ○ labile phosphate produced.
Components in the main compartment: resting cell suspension 0.5 ml. (*ca.* 6 mg. nitrogen/ml.), sodium lactate 300 μmoles, $MgCl_2$ 10 μmoles, NaF 20 μmoles, inorganic orthophosphate 4 μmoles, ^{32}P 4×10^4 c.p.m., pH 8.0 Tris-buffer (final concentration 0.025 *M*). Total volume 3 ml. with H_2O.
Side arm: $NaNO_2$, 60 μmoles.
Center well: 20% KOH 0.2 ml.

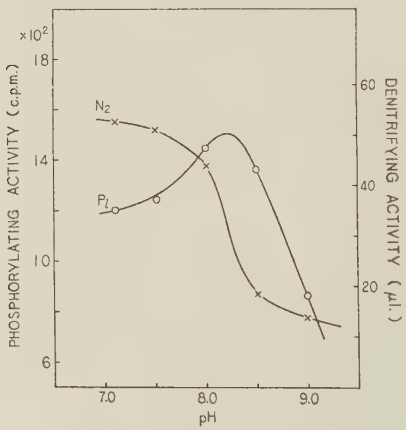


FIG. 2. Effect of pH on denitrification and phosophorylation by resting cells.
× denitrifying activity, ○ phosphorylating activity.
Experimental conditions were the same as indicated in Fig. 1.
Reaction time was 10 minutes.
Resting cell suspension 0.5 ml. (*ca.* 3.1 mg. nitrogen /ml.).

of ratio P (moles)/N (atoms), also continued to increase in this range of phosphate concentration and maximal ratio obtained was 0.05.

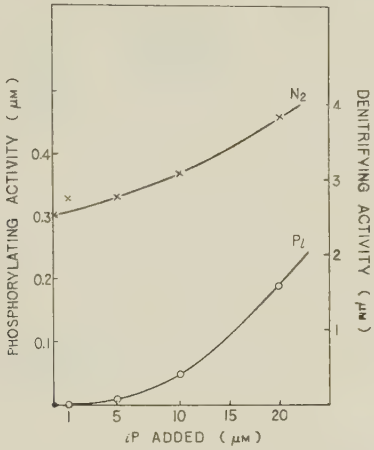


Fig. 3. Influence of phosphate concentration on the denitrification and phosphorylation by resting cells.
× denitrifying activity, ○ phosphorylating activity. Experimental conditions were the same as indicated in Fig. 1.

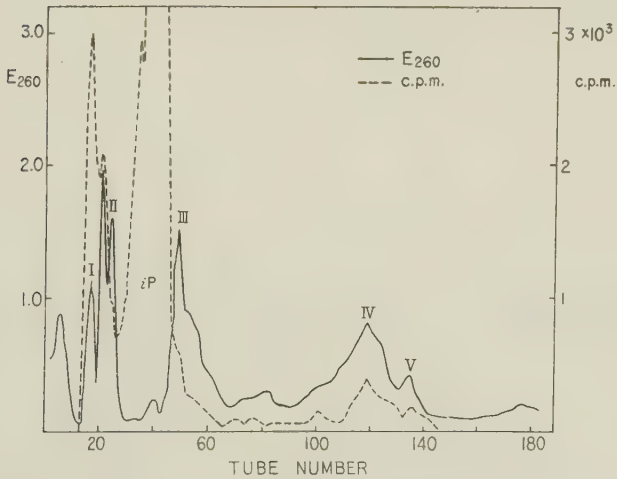


Fig. 4. Gradient chromatography of the acid-soluble extract of denitrifying bacteria with "formic acid system." For experimental conditions see the text.

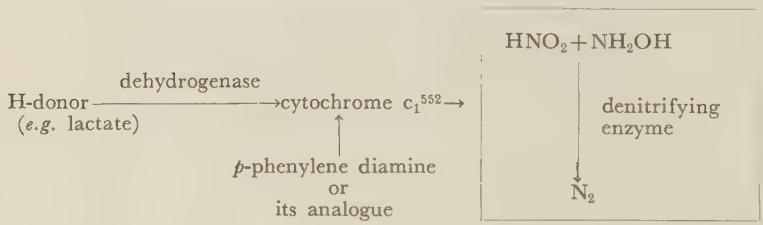
Analysis of Nucleotides incorporating Phosphate—After a certain duration (10 minutes) of denitrifying reaction, the bacterial cells were extracted with

PCA, and the extract obtained was fractionated using a column of Dowex-1 ion exchange resin by the gradient elution system as described in the section of methods. Ultraviolet absorption and radioactivity of the chromatographic fractions are shown in Fig 4. Fractions indicated on the graph as Peak I, II, III, IV and V were tentatively identified on the basis of their 280 mμ./260 mμ. ratio and ultraviolet absorption curves as cytidine monophosphate+an unidentified substance, adenosine monophosphate, its diphosphate and triphosphate, and guanosine triphosphate, respectively. Other peaks were considerably overlapped by each other in this experiment. As may be seen from the result, all peaks mentioned above, except for Peak I, coincided with the peaks for radioactivity, indicating that P^{32}-labelled orthophosphate was incorporated into all of these nucleotides nearly at the same rate in the cell.

Phosphorylation in the Intermediate Steps of Denitrification—Recently, a possible mechanism of the denitrifying system was formulated by Iwasaki and Mori in our laboratory as shown in Scheme 1 (*8*, *9*).

SCHEME 1

Denitrifying Pathway Formulated by Iwasaki and Mori



It was further investigated whether orthophosphate was incorporated into acid-soluble fraction in each of the intermediate oxidation steps or in partial processes of the denitrifying pathway. Activity of phosphorylation was measured through the same procedure as in the lactate-nitrite system, with dimethyl-*p*-phenylene diamine or hydroxylamine as the hydrogen donor and sodium nitrite as the hydrogen acceptor. Preliminary results were shown in Table I. These results indicate, although only in qualitative terms at present, that the coupling between denitrification and phosphorylation occurred in at least one of the electron transporting steps in the reaction between hydroxylamine and nitrite which was presumed, at present, to be the principal reaction (*8*) or in a reaction process of dimethyl-*p*-phenylene diamine with nitrite (*10*). It would be worth mentioning that the underlying electron transferring mechanisms of these reactions has been inferred to involve the oxidation-reduction of certain cytochromes (*9*).

TABLE I

Phosphorylation Coupled with the Intermediate Steps in the Denitrifying Pathway

H-Donor H-Acceptor	Denitrifying Activity, μl.	Phosphorylating Activity, c.p.m.
Lactate $NaNO_2$	74.3	740
NH_2OH $NaNO_2$	202.5	1346
DMPPD[1)] $NaNO_2$	182.0	3840

Experimental conditions were the same as shown before, except that the amount of inorganic orthophosphate was 20 μmoles in this experiment.

1) dimethyl-*p*-phenylene diamine

SUMMARY

1. Using whole cell suspensions of a denitrifying bacterium and P^{32} as a tracer, it was demonstrated that phosphorylation takes place coupling with the denitrifying reaction under the anaerobic condition.

2. The dependencies of both nitrogen evolution and phosphate incorporation on pH, length of reaction time and concentration of phosphate were investigated.

3. Soluble compounds extracted by perchloric acid were analysed by the chromatographic fractionation and it was demonstrated that along with the denitrification lasting for only 10 minutes, P^{32} was incorporated into almost all nucleotides appeared in the chromatogram.

4. Some intermediate steps in the denitrifying pathway were also found to evoke the process of phosphorylation.

REFERENCES

(*1*) Sato, R., *Scienca Revuo.*, **2**, 122 (1950)
(*2*) Takahashi, H., Taniguchi, S., and Egami, F., *J. Biochem.*, **43**, 223 (1956)
(*3*) Ishimoto, M., Egami, F., "Symposium on Origin of Life on Earth," P. 554 (1959), Nat. Acad. Sc. USSR. (in Russian)
(*4*) Iwasaki, H., and Mori, T., *J. Biochem.*, **42**, 375 (1955).
(*5*) Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201**, 235 (1953)
(*6*) Nielsen, S. O., and Lehninger, A. L., *J. Biol Chem.*, **215**, 555 (1955)
(*7*) Hurlbert, B., Robert, S. H., Brumm, F. A., and Potter, R. V., *J. Biol. Chem.*, **209**, 23 (1954)
(*8*) Iwasaki, H., and Mori, T., *J. Biochem.*, **45**, 133 (1958)
(*9*) Iwasaki, H., *J. Biochem.*, **47**, 174 (1960)
(*10*) Iwasaki, H., Matsubayashi, R., and Mori, T., *J. Biochem.*, **43**, 295 (1956)

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METABOLISM OF L-LYSINE BY BACTERIAL ENZYMES

IV. δ-AMINOVALERIC ACID-GLUTAMIC ACID TRANSAMINASE

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We reported previously that in *Pseudomonas* L-lysine was oxidatively decarboxylated forming δ-aminovaleric acid and that this then underwent a transamination reaction with α-ketoglutaric acid (*1*). Roberts also found that δ-aminovaleric acid could be transaminated in mouse liver, *Aspergillus* and *E. coli* (*2*). However, none of these reports gave details of the transaminases, such as purification, substrate specificity or identification of the end products. Baxter and Roberts recently reported on the purification of γ-aminobutyric acid-glutamic acid transaminase from beef brain (*3*). This enzyme has a rather low specificity for amino group donors being active with β-alanine and δ-aminovaleric acid as well as γ-aminobutyric acid.

The present paper describes the purification of δ-aminovaleric acid-glutamic acid transaminase from *Pseudomonas*. This enzyme is very specific for δ-aminovaleric acid. Glutaric semialdehyde was identified as the end product.

METHODS AND MATERIALS

Preparation of the Acetone Powder of Pseudomonas Adapted to L-Lysine—The bacterial strain of *Pseudomonas* used for the present work and the method of preparation of the acetone powder were as described previously (*1*).

Assay Method—Thirty μmoles of δ-aminovaleric acid, 40 μmoles of α-ketoglutaric acid, 20 μmoles of phosphate buffer (pH 8.0) and 0.2 ml. of the enzyme solution were incubated in a total volume of 2 ml. in a test tube at 30° for one hour. Then the reaction mixture was deproteinized by the addition of 0.3 ml. of 50 per cent perchloric acid. The supernatant was adjusted to pH 5.0 with 5 *N* potassium hydroxide. 1.5 ml. of the supernatant was incubated in a Warburg manometer at 30° with 60 μmoles of acetate buffer (pH 5.0) and 0.2 ml. of L-glutamic acid decarboxylase in a total volume of 2 ml. The L-glutamic acid formed was calculated from the amount of carbon dioxide produced. The enzymic activity was expressed as μmoles of glutamic acid formed per hour.

The reversibility of the transamination was measured by incubating a reaction mixture similar to the above, but containing glutaric semialdehyde and L-glutamic acid in place of the δ-aminovaleric acid and α-ketoglutaric acid. The L-glutamic acid remaining at the end of the reaction was measured in the same way as described above, by carbon

dioxide production. Protein was measured by Folin's method (*6*).

δ-Aminovaleric acid hydrochloride and glutaric acid semialdehyde ethyl ester were kindly given by Prof. S. Akabori of this Institute. Glutaric semialdehyde was prepared from its ester by hydrolyzing it at 100° for 30 minutes with Dowex 50 (H^+ form).

The acetone powder of *E. Coli* used as the L-glutamic acid decarboxylase was prepared by Najjar's method (*4*) and 50 mg. of the powder was suspended in 1 ml. of water before use as described above.

Pyridoxal phosphate was obtained from California Foundation.

RESULTS

Purification of the Transaminase—Ten g. of acetone powder of *Pseudomonas* adapted to L-lysine was homogenized with 400 ml. of 0.02 *M* phosphate buffer (pH 7.4) and stored in a cold room overnight. One tenth volume of 5 per cent streptomycin was slowly added to the supernatant (Extract) with stirring, and after 10 minutes the precipitate was removed by centrifugation. The supernatant (Streptomycin treatment) was brought to 55 per cent saturation with solid ammonium sulfate, and the mixture was stirred for 30 minutes. Throughout this step the pH was kept at 7.5 by addition of 10 per cent ammonia solution. After centrifugation further ammonium sulfate was added to the supernatant to 75 per cent saturation. The resulting precipitate was dissolved in 30 ml. of 0.007 *M* phosphate buffer and dialyzed against 2 liters of the same buffer overnight. The dialyzate (Ammonium sulfate fraction I) was acidified at 0° with 1 *N* acetic acid. Material precipitating at pH 5.0 was discarded. Ca-phosphate gel was added to the supernatant (Acid treatment) in the ratio of one part gel (dry weight) to two

TABLE I

Purification of δ-Aminovaleric Acid -Glutamic Acid Transaminase

The specific activity was expressed as μmoles of glutamate formed per mg. protein per hour.

	Specific activity	Total activity
	μmol./mg./hr.	μmol./hr.
Extract	0.28	1,476
Streptomycin treatment	0.78	1,500
Ammonium sulfate fraction I	2.45	1,010
Acid treatment	2.67	980
Ca-gel treatment	4.02	665
Ammonium sulfate fraction II	6.19	509

parts protein. The pH was adjusted to 6.0 and the mixture stirred for 20 minutes. After centrifugation, the supernatant (Ca-gel treatment) was fractionated again with ammonium sulfate. The fraction precipitating between 50 and 80 per cent saturation was dissolved in 5 ml. of phosphate buffer and dialyzed as before. The dialyzate (Ammonium sulfate fraction II) was used

as the enzyme preparation. Throughout the purification procedure, the temperature was kept below 4°. Centrifugation was carried out at 15,000× *g* for 15 minutes in each case.

The typical yields and specific activities obtained at each step of purification are shown in Table I. At the final step the yield was 30 per cent with a 20 fold increase in specific activity.

The relations between the transaminase activity and protein concentration and incubation time are shown in Fig. 1 and 2, respectively.

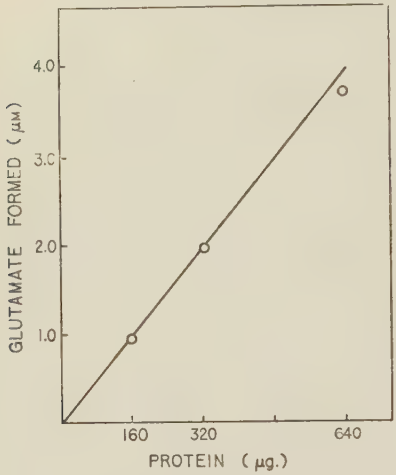


Fig. 1. Effect of concentration of protein on the transaminase activity.

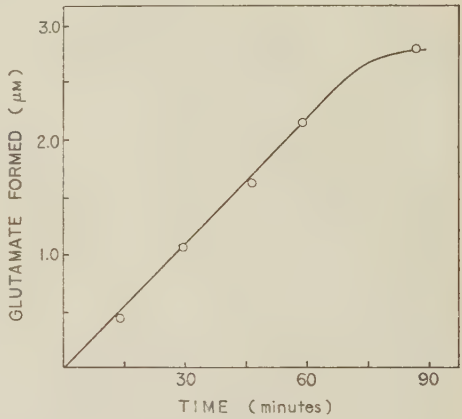


Fig. 2. Effect of incubation period on the transaminase activity.

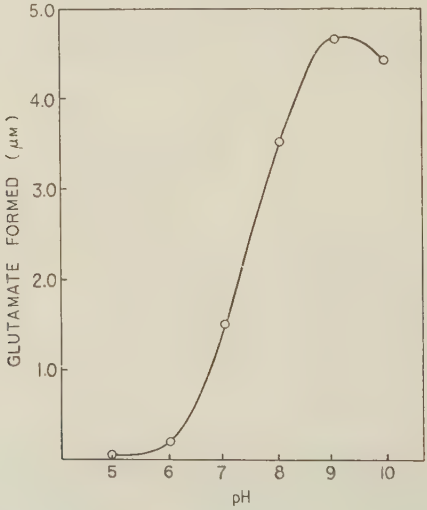


Fig. 3. Effect of pH on transaminase activity. Acetate buffer (pH 5), phosphate buffer (pH 6, 7 and 8), and borate buffer (pH 9 and 10) were used respectively.

Optimal pH—The transaminase showed optimal activity at pH 9 with a rather sharp decrease in activity on the acid side (Fig. 3).

Substrate Specificity—Of the possible amino donors examined, significant activity was observed only with δ-aminovaleric acid, as shown in Table II.

TABLE II

Specificity of the Transaminase

Substrate	Amount	L-Glutamic acid formed
	μmoles	μmoles
δ-Aminovaleric acid	20	2.68
Glycine	10	0.25
β-Alanine	20	0.36
γ-Aminobutyric acid	20	0.38
DL-Ornithine	20	0.53
L-α-γ-Diaminobutyric acid	10	0.12

γ-Aminobutyrate, α-γ-diaminobutyrate, ornithine, glycine and β-alanine were without effect.

As an amino acceptor pyruvic acid was tested in place of α-ketoglutaric acid. 0.2 ml. of the resulting incubation mixture after deproteinization, was applied to a paper chromatogram and developed in butanol-acetic acid-water, (4 : 1 : 1, v/v). No ninhydrin positive spot could be detected in the position of alanine (R_f 0.30). This suggests that the transaminase is specific for α-ketoglutarate.

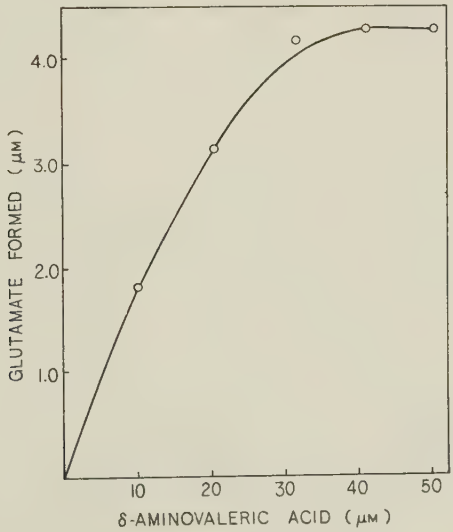


FIG. 4. Transaminase activity with different concentration of δ-aminovaleric acid.

Kms of δ-Aminovaleric Acid and α-Ketoglutaric Acid—The transaminase activity with different concentrations of δ-aminovaleric acid and α-ketoglutaric acid are shown in Fig. 4 and Fig. 5 respectively. From these results the

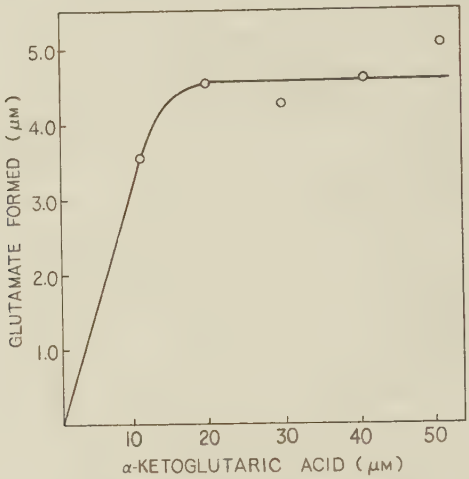


FIG. 5. Transaminase activity with different concentrations of α-ketoglutaric acid.

Kms were calculated as $1\times10^{-2}M$ and $8\times10^{-3}M$ respectively. The Km for glutamic acid in the reverse reaction was found to be $4.1\times10^{-3}M$.

Reversibility of the Reaction—Fifty μmoles of glutaric semialdehyde, 50 μmoles of L-glutamic acid, 50 μg. of pyridoxal phosphate, 60 μmoles of phosphate buffer and 0.7 ml. of enzyme solution in a total volume of 2 ml. were incubated for one hour at 30°. Two tenth ml. of the reaction mixture after deproteinization was chromatographed on paper in the solvents shown in

TABLE III

R_f of δ-Aminovaleric Acid and the Compound in the Reaction Mixture

No.	Solvent	δ-Aminovalerate	Compound in the Reaction Mixture		Glutamate
1.	Butanol : Acetic acid : H_2O 4 : 1 : 1	0.52	0.52	0.30	0.28
2.	*iso*-Propanol : H_2O : NH_3 120 : 20 : 10	0.10	0.10	0.00	0.00
3.	Ethanol : NH_3 : H_2O 180 : 10 : 10	0.29	0.30	0.05	0.05
4.	Phenol : H_2O 160 : 40	0.84	0.85	0.37	0.35

Table III. The ninhydrin positive spots which appeared had R_f values coinciding with those of an authentic sample of δ-aminovaleric acid. When

either substrate was omitted from the reaction mixture, no ninhydrin positive spot could be detected. By analysis of the spots it was found that 8.5 μmoles of the L-glutamic acid had been consumed during the reaction.

Identification of Glutaric Semialdehyde as a Reaction Product—Fifty μmoles of δ-aminovaleric acid, 50 μmoles of α-ketoglutaric acid, 50 μg. of pyridoxal phosphate, 60 μmoles of phosphate buffer and 0.7 ml. of the enzyme preparation, in a total volume of 2 ml. was incubated for one hour at 30°. Then an equal volume of a 0.3 per cent solution of 2,4-dinitrophenylhydrazine in 2*N* HCl was added to the deproteinized mixture. After several hours the mixture was centrifuged. The crystals which precipitated were mainly the hydrazone of α-ketoglutarate. The supernatant was shaken vigorously with 1 ml. of ethyl acetate and 0.1 ml. of the ethyl acetate layer was chromatographed as shown in Table IV. The hydrazones of both α-ketoglutarate and glutaric

TABLE IV

R_f of the Hydrazones of Glutaric Semialdehyde and of the Compound in the Reaction Mixture

Solvent	Glutaric semialdehyde	Compound in the reaction mixture	α-Ketoglutarate
iso-Propanol : H_2O : NH_3 120 : 20 : 10	0.40	0.39	0.1
n-Butanol : Ethanol : NH_3 : H_2O 35 : 5 : 0.5 : 10	0.50	0.52	0.1
Ethanol : Petroleum ether 40 : 10	0.99	0.95	0.52
tert-Amyl alcohol : Ethanol : H_2O 25 : 5 : 20	0.93	0.90	0.70

semialdehyde were also applied to the paper as controls. The R_f of the yellowish brown spot from the reaction mixture coincided with that of the hydrazone of glutaric semialdehyde. No hydrazone spot could be detected when either δ-aminovaleric acid or α-ketoglutaric acid was omitted from the reaction mixture. Aliquots of the reaction mixture were taken to measure the amount of L-glutamic acid formed, and this was estimated as 4.9 μmoles.

Inhibitors and Cofactors—To avoid the complication that the inhibitor used might react with L-glutamic acid decarboxylase, L-glutamic acid was determined quantitatively by paper chromatography using solvent No. 1 given in Table III. After development, the area of the paper corresponding to the position of L-glutamic acid was cut out and eluted with 1 ml. of water. The amino acid concentration was measured with ninhydrin (*5*).

As shown in Table V, *p*-chloromercuribenzoate inhibited the reaction markedly, suggesting that an SH-group may be involved in the transaminase reaction. Although hydroxylamine and semicarbazide also inhibited the reaction, it is uncertain whether these carbonyl reagents showed enzyme inhibition or whether they had an effect by combining with the α-ketoglutaric acid and thus decreasing the concentration of this substrate.

It is clear that pyridoxal phosphate is a cofactor for the transaminase, but without its addition to the reaction mixture the enzyme preparation used in this study showed fair activity. Extensive dialysis of the enzyme

TABLE V

The Effect of Inhibitors on the Transaminase Reaction

Inhibitor	Concentration	Glutamate formed
	M	μmole
—	—	3.1
p-Chloromercuribenzoate	1×10^{-4}	0.8
Ethylenediamine tetracetate	1×10^{-3}	3.3
Hydoxylamine	,,	0.2
Semicarbazide	,,	0.5
Sodium azide	,,	3.0

against $7\times10^{-2}M$ phosphate buffer containing $1\times10^{-3}M$ hydroxylamine for 14 hours and then against a similar buffer without hydroxylamine for 3 hours resulted in a preparation which was inactive without the addition of pyridoxal phosphate (Table VI).

TABLE VI

Effect of Pyridoxal Phosphate on the Transaminase Activity

Dialysis	Pyridoxal phosphate	Glutamate formed
	μg.	μmoles
Before	—	3.2
	50	3.1
After	—	0.3
	50	2.4
	100	2.9

DISCUSSION

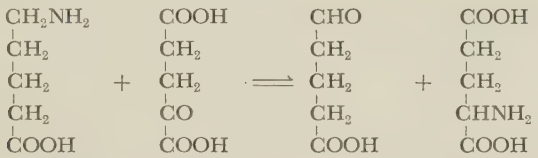
The formation of δ-aminovaleric acid from proline was discovered by Stickland in 1935 using *Clostridium* (*9*), and proline reductase was isolated by Stadtman *et al.* (*8*). The formation of this ω-amino acid from lysine was also suggested by Neuberger and Sanger in 1944 (*18*). and Suda *et al.* found in 1954 that L-lysine was converted oxidatively to δ-aminovaleric acid in *Pseudomonas* (*1*). Subsequently they isolated and purified L-lysine oxidase from this bacteria (*7*). Rothstein and Miller, however, found that in animals L-lysine was metabolized maninly to pipecolic acid (*17*).

In 1954 Suda *et al.* working with *Pseudomonas* (*1*) and Roberts work-

ing with *E. Coli*, *Aspergyllus* and mouse liver (*2*), reported on the transaminase of δ-aminovaleric acid. Neither paper however, gave details of the transaminase such as substrate specificity or identification of the end product. Baxter and Roberts (*3*) have purified γ-aminobutyrate-glutamate transaminase from beef brain, while Nishizuka *et al.* (*10*) have obtained β-alanine -alanine transaminase in a purified form from *Pseudomonas fluorescens.* Both transaminases have a rather low substrate specificity and δ-aminovaleric acid as well as β-alanine and γ-aminobutyric acid can be transaminated.

The transaminase reported here, however, has a very strict specificity for δ-aminovaleric acid, and is thus distinct from the two enzymes mentioned above.

The reaction proceeds as follows:



Glutaric semialdehyde and glutamic acid were identified as the end products.

Scott and Jacoby (*11*) isolated γ-aminobutyric acid-glutamic acid transaminase from *Pseudomonas fluorescens.* This had a very similar nature to the enzyme reported here but δ-aminovaleric acid could not be used as a substrate.

With the present enzyme preparation, the observation that prolonged dialysis resulted in a preparation which was inactive without the addition of pyridoxal phosphate strongly indicates that pyridoxal phosphate is a cofactor.

Studies on the metabolism of δ-aminovaleric acid began when Keil isolated β-aminomethylethylketone from dog urine after administration of δ-aminovaleric acid (*12*). This amino acid has also been shown to be neither glycogenic nor ketogenic (*13*). Rothstein and Miller showed that δ-aminovaleric acid is converted to glutaric and glutamic acids in rats *in vivo* (*14*). Since glutaric acid can be converted to α-ketoglutaric acid (*15*, *16*), the formation of glutamic acid is probably *via* α-ketoglutaric acid.

Rothstein and Miller suggested that the metabolic pathway of δ-aminovaleric acid was as follows:

δ-aminovaleric acid ⟶ glutaric semialdehyde (1)
glutaric semialdehyde ⟶ glutaric acid (2)

The present study shows the existence of reaction (*1*). Reaction (*2*) was recently demonstrated in an extract of an acetone powder of the same *Pseudomonas* by showing that pyridine nucleotide was reduced when glutaric semialdehyde was added. A study of this hydrogenase is now in progress, and will be reported soon.

SUMMARY

1. δ-Aminovaleric acid-glutamic acid transaminase was isolated from *Pseudomonas* and purified.

2. This enzyme catalyzes the following reaction:

$$\begin{array}{ccc} \delta\text{-aminovaleric acid} & & \text{glutaric semialdehyde} \\ + & \rightleftharpoons & + \\ \alpha\text{-ketoglutaric acid} & & \text{L-glutamic acid} \end{array}$$

Substrate specificity was restricted to α-aminovaleric acid and α-ketoglutaric acid. None of the several amino acids nor pyruvic acid tested were inactive. Glutaric semialdehyde was identified as the product of the forward reaction and δ-aminovaleric acid as that of the reverse reaction.

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REFERENCES

(*1*) Suda, M., Kamahora, T., and Hagihira, H., *Med. J. Osaka Univ.*, **5**, 119 (1954)
(*2*) Roberts, E., *Arch. Biochem. Biophys.*, **48**, 395 (1954)
(*3*) Baxter, C. F., and Roberts, E., *J. Biol. Chem.*, **233**, 1135 (1958)
(*4*) Najjar, V. A., "Methods in Enzymology", edited by Colowick, S., and Kaplan, N. O., Academic Press, New York, Vol. **II**., 185 (1955)
(*5*) Cocking, E. C., and Yemm, E. W., *Biochem. J.*, **58**, xii (1954)
(*6*) Layne, E., "Methods in Enzymology", edited by Colowick, S., and Kaplan, N. O., Academic Press, New York, Vol. **III**., 447 (1957)
(*7*) Hagihira, H., Hayashi, H., Ichihara, A., and Suda, M., *J. Biochem.*, **48**, 267 (1960)
(*8*) Stadtman, T. C., and Elliot, P., *J. Biol. Chem.* **228**, 983 (1957)
(*9*) Stickland, L. H., *Biochem. J.*, **29**, 288 (1935)
(*10*) Nishizuka, Y., Takeshita, M., Kanazuna, F., Tachibana, M., Kuno, S., and Hayashi, O., presented at the 11th Meeting of Symposia on Enzyme Chemistry, Kyoto, Japan (1959)
(*11*) Scott, E. M., and Jakoby, W. B., *J. Biol. Chem.*, **234**, 932 (1959)
(*12*) Keil, W., *Z. physiol. Chem.*, **207**, 248 (1932)
(*13*) Corley, R. C., *J. Biol. Chem.*, **70**, 99 (1926)
(*14*) Rothstein, M., and Miller, L. L., *Arch. Biochem. Biophys.*, **54**, 1 (1955)
(*15*) Rothstein, M., and Miller, L. L., *J. Biol. Chem.*, **211**, 859 (1954)
(*16*) Hobbs, D. C., and Koeppe, R. E., *J. Biol. Chem.* **230**, 655 (1958)
(*17*) Rothstein, M., and Miller, L. L., *J. Biol. Chem.*, **206**, 243 (1954)
(*18*) Neuberger, A., and Sanger, F., *Biochem. J.*, **38**, 119 (1944)

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MANOMETRIC DETERMINATION OF L- AND D-LYSINE BY BACTERIAL L-LYSINE OXIDASE AND ITS RACEMASE

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Specific amino acid decarboxylases have often been used as convenient tools for the determination of various amino acids (*1*). Among a number of L-amino acids examined, Gale established a method for the determination of L-lysine, using an acetone powder of *Bacterium cadaveris* (*2*). Recently the authors have found a species of *Pseudomonas* which adaptively metabolizes D- and L-lysine (*3*).

An acetone powder of this bacteria contains L-lysine oxidase and lysine racemase. The L-lysine oxidase is very specific for L-lysine, metabolizing it to δ-aminovaleric acid with a simultaneous equimolecular oxygen consumption (*4*). Hydroxylamine completely inhibits the action of the racemase, but not that of the oxidase (*5*).

The present paper describes the manometric, differential determination of L- and D-lysine by this acetone powder of *Pseudomonas* which contains L-lysine oxidase and lysine racemase.

METHODS

Preparation of the Acetone Dried Powder of Pseudomonas—The strain of *Pseudomonas* used for this work was the same as that used in the previous work (*3*). The bacteria grown in

Table I

DL-*Lysine Medium*

Compound	g. per liter
Na_2HPO_4	3
KH_2PO_4	2
DL-Lysine · HCl	2
NaCl	1
$MgSO_4$	0.05

Final pH adjusted to 7.4 with NaOH

peptone-bouillon medium were harvested and inoculated into 10 liters of DL-lysine medium, as shown in Table I. After maximal growth had been obtained, the bacteria

were harvested in a Sharples centrifuge, and washed with 100 ml. of cold water. They were then mixed with 2 liters of cold acetone (−15°) with vigorous stirring. After 15 minutes, the bacterial cells were recovered through a Buchner's funnel. The packed cell cake was rapidly air-dried and then stored in a desiccator *in vacuo*.

One gram of the acetone powder was suspended in 40 ml. of water. After homogenization, 0.5 ml. of the suspension per Warburg manometer vessel was used for lysine assay.

The acetone powder could be prepared from resting cells adapted to DL-lysine, as reported previously (*3*). The L-lysine oxidase activity was the same in both preparations.

Assay Method—A conventional Warburg manometer was used to assay the lysine. The main compartment of the vessels contained; 0.5 ml. of the acetone powder suspension and 60 μmoles of phosphate buffer (pH 8.0), and the side chamber; 0.1 to 0.5 ml. of substrate. The total volume was made up to 2 ml. with distilled water.

When L-lysine was to be measured, 40 μmoles of hydroxylamine was added to the contents of the main chamber.

Oxygen consumption was measured at 30°. After completion of the reaction, the total amount of lysine was calculated from the amount of oxygen consumed in the absence of hydroxylamine, and the amount of L-lysine from that consumed in the presence of hydroxylamine. The amount of D-lysine was deduced by subtracting the amount of L-lysine from the total lysine.

RESULTS AND DISCUSSION

Preliminary Experiments—As reported previously, our L-lysine oxidase is very specific for L-lysine and was without activity with fifteen other amino acids tested (*4*). This was also true with a preparation of the acetone powder of cells adapted to DL-lysine. The oxygen consumption was proportional and equimolar to the L-lysine concentration, as shown in Fig. 1. The concentration of hydroxylamine used completely inhibits the racemase (*5*), but has no effect on the L-lysine oxidase activity (*4*, *5*). The results shown in Fig. 1 are repeatable with errors of ± 0.5 μmoles. Therefore the method is good except when concentrations of less than 2 μmoles of lysine are to be measured.

Application of the Method to the Determination of Lysine in Rat Urine—There have been a number of reports showing that D-lysine cannot support the growth of rats (*6*) and mice (*7*). Miller and his colleagues showed by perfusion (*8*) and *in vivo* experiments (*9*) that D-lysine is not significantly metabolized in rat liver.

We studied the distribution of D-lysine in the body of rats after it had been injected intramuscularly. One hundred and fifty g. male albino rats were fed on a diet of 20 per cent casein. Each animal received an intramuscular injection of 200 mg. of DL-lysine -HCl (1100 μmoles) in 1 ml. of 0.9 per cent saline solution. After this, feeding was discontinued and the urine was collected in flasks containing 1 ml. of 1 *N* HCl. D-Lysine and L-lysine were measured in the 24 hour urine samples. Some of the rats were killed after 24 hours. The viscera, liver and the carcass were homogenized in a Waring blender with 5 per cent perchloric acid solution. The extracts were neutralized and centrifuged. The supernatants were con-

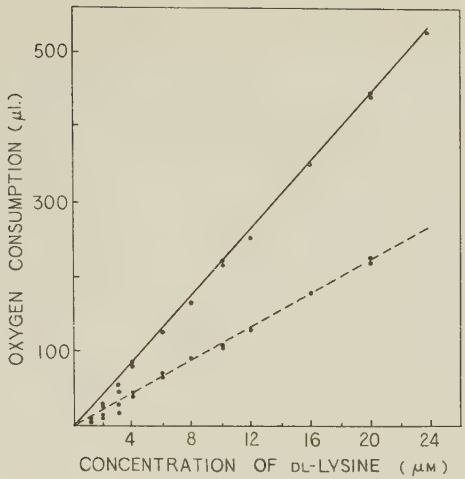

FIG. 1. Oxygen consumption with different concentration of DL-lysine.

At each of the concentrations indicated, L-lysine (dotted line) was measured in the presence of and DL-lysine (solid line) in the absence of hydroxylamine. D-lysine was calculated from the difference between these two concentrations, as described in the text.

TABLE II

Distribution of D- *and* L-*Lysine 24 Hours after Injection of* DL-*Lysine*

Number of Rats tested	3[a]		6		3[b]	
Lysine	D	L	D	L	D	L
Urine-1st day	0	41	255	75	278	56
Liver	—	—	—	—	32	15
Blood and Viscera	—	—	—	—	15	54
Carcass	—	—	—	—	63	70
Urine-2nd day	—	—	90	23	—	—
Urine-3rd day	—	—	63	36	—	—

Figures represent the mean values (in μmoles) in the rats tested.

a) Control rats.

b) Rats were killed after 24 hours.

centrated to small volume and the amounts of D- and L-lysine measured. The results are shown in Table II. It was found that about 50 per cent of the D-lysine injected was excreted in the urine within 24 hours after its

injection, Lesser amounts were excreted during the subsequent two or three days. The amount of L-lysine in the urine did not increase significantly over the control value. Fifty per cent of the injected D-lysine was recovered in the urine within 24 hours and about 20 per cent in the body of the rats. These results are compatible with those reported previously for D-lysine-C^{14} (*9*) ane D-lysine-N^{15} (*10*).

It is very interesting that over 80 per cent of the L-lysine injected could not be found in either the urine or nonprotein contents of the body. It had probably either been metabolized or incorporated into body protein. The D-lysine unaccounted for may have been metabolized, but its disappearance was probably due to technical error. Ratner *et al.* found that 40 per cent of D-lysine nitrogen was metabolized (*10*), whereas Rothstein and Miller claim that D-lysine is neither metabolized nor incorporated into proteins (*9*).

SUMMARY

1. A manometric method for the differential determination of L- and D-lysine has been developed, using a preparation of the acetone powder of *Pseudomonas* bacteria, which contains L-lysine oxidase and its racemase. The method is quite specific for lysine and gives an error of $\pm 0.5\ \mu$moles.

2. Using this method the fate of DL-lysine following its injection into rats was investigated. Seventy per cent of the D-lysine and less than 20 per cent of the L-lysine could be detected in the urine and non-protein contents of the body 24 hours after its injection.

REFERENCES

(*1*) Najjar, V. A., "Methods in Enzymology" edited by Colowick, S., and Kaplan, N. O., Academic Press, Vol. **III**, 462 (1957)
(*2*) Gale, E. F., *Biochem. J.*, **39**, 46 (1945)
(*3*) Suda, M., Kamahora, T., and Hagihira, H., *Med. J. Osaka Univ.*, **5**, 119 (1954)
(*4*) Hagihira, H., Hayashi, H., Ichihara, A., and Suda, M., *J. Biochem.*, **48**, 267 (1960)
(*5*) Ichihara, A., Furuya, S., and Suda, M., *J.Biochem.*, **48**, 277 (1960)
(*6*) Berg, C. P., *J. Nutrition*, **12**, 671 (1936)
(*7*) Totter, J. R., and Berg, C. P., *J. Biol. Chem.*, **127**, 375 (1939)
(*8*) Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F., *J. Exptl. Med.*, **94**, 431 (1951)
(*9*) Rothstein, M., Bly, C. G., and Miller, L. L., *Arch. Biochem. Biophys.*, **50**, 252 (1954)
(*10*) Ratner, S., Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **147**, 549 (1943)

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EFFECTS OF KANAMYCIN ON THE P^{32}-PHOSPHATE AND S^{35}-SULFATE INCORPORATION INTO THE KANAMYCIN-SENSITIVE AND KANAMYCIN-RESISTANT CELLS OF MYCOBACTERIUM AVIUM

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There is little knowledge of the mechanism involved in the action of kanamycin. Tsukamura and Tsukamura (*1*) reported that kanamycin precipitates both nucleic acids, deoxypentose nucleic acid (DNA) and pentose nucleic acid (RNA), especially the latter one. Aoki, Hayashi and Ito (*2*) reported on the inhibition of the adaptive oxidation of benzoic acid, niacin and malonic acid by kanamycin in *Mycobacterium avium*. These results suggested that kanamycin may inhibit the function of RNA. Isotopic studies, which are reported in the present paper, seems to support this hypothesis.

MATERIALS AND METHODS

Strain—Mycobacterium avium, strain Jucho, was used throughout. The Kanamycin-resistant strain used was isolated from the parent sensitive strain by multi-step selection with increasing concentrations of kanamycin. It was resistant to 1 μg. of kanamycin per ml.

Eight day-old cultures of both sensitive and resistant strains grown in modified Sauton medium, in which sodium glutamate was substituted for asparagine, were used as the sources of cell suspensions. This culture age corresponded to the stationary growth phase.

Preparation of Cell Suspensions—The cells grown were washed three times with physiological saline, homogenized by shaking with glass beads and suspended in 0.033 *M* phosphate buffer, pH 7.1, or in phosphorus-deficient medium consisted of 20 ml. of glyceriol, 2.0 g. of sodium glutamate, 1.0 g. of sodium citrate, 0.25 g. of magnesium sulfate, 0.25 g. of pottassium chloride, 0.025 g. of ferrous sulfate, and 980 ml. of distilled water (pH 7.0). To these cell suspensions, radioisotopes and kanamycin sulfate (Meiji Seika Co.) were added to obtain appropriate final concentrations.

Each reaction mixture (4.0 ml.) was contained in a centrifugation tube and incubated statically at 37°. The amounts of cells in the reaction mixture were expressed as mg., wet weight, of cells.

Fractionation of Phosphorus Componuds—After an appropriate period of incubation (static culture), the cells were collected by centrifugation and washed three times in distilled water. The last washing fluid contained practically no P^{32} radioactivity. The cells were fractionated according to the procedure of Schneider (*3*).

Fractionation of Sulfur Compounds—The cells were collected by centrifugation and

washed four times in distilled water. The cells were then extracted three times with 10 per cent trichloroacetic acid (TCA). These extracts were designated as the TCA-soluble fraction. The following fractionation was conducted according to the procedure of Schneider (*3*). The protein fraction obtained by this fractionation was designated as the TCA-insoluble fraction.

Isotopic Techniques—Each 0.2 ml. of fractions was placed onto stainless steel planchets and heated to dryness. The radioactivity was counted by a Geiger Mueller counter. P^{32}-orthophosphate supplied by the Radiochemical Center, Amersham, England, and S^{35}-sulfate supplied by the Commissariat a l'Energiew Atomique, Saclay, France, were used. The amounts of isotopes were expressed as "counts per minute" (c.p.m.) per 1 μg. of DNA. The amount of DNA remained unchanged throughout the experiments.

Determination of the Amounts of DNA and RNA—The amounts of DNA and RNA were determined in the nucleic acid fraction by the diphenylamine reaction (*4*) and the oricinol-HCl reaction (*5*), respectively.

RESULTS

Inhibition of the Incorporation of Radioactive Phosphorus (P^{32}) into the Nucleic Acid and into the Protein Fractions—Incorporations of P^{32} into the nucleic acid and into the protein fractions of the parent sensitive strain were inhibited by the presence of only 1 μg. of kanamycin per ml. (Table I). Incorporation of P^{32} into the acid-soluble fraction was not inhibited significantly.

TABLE I

Distribution of Radioactivity from Incorporated P^{32} in Cellular Fractions of Parent Sensitive Strain

Reaction System[1)]	Time of Incubation	Radioactivity as c.p.m. per 1 μg. of DNA			
		Fraction:			
		Acid-Soluble	Phosphatide	Nucleic Acid	Protein
Control	1 Hour	8.30	0.27	8.90	0.44
KM 1 μg./ml.		6.50	0.22	8.00	0.44
Control	2 Hour	10.2	0.26	12.2	0.83
KM 1 μg./ml.		9.82	0.22	10.4	0.42
Control	3 Hour	10.4	1.40	14.0	1.00
KM 1 μg./ml.		10.7	0.42	9.00	0.46
Control	5 Hour	9.08	0.66	20.6	1.88
KM 1 μg./ml.		7.78	0.16	9.30	0.32

1) Reaction system (4.0 ml.): Cells suspended in phosphorus-deficient medium, 45 mg./ml.; P^{32}-orthophosphate, 1.5 μc./ml.; Kanamycin sulfate (KM), 0 or 1 μg./ml., (in final concentrations)

Inhibition of the Incorporation of Radioative Sulfur (S^{35}) into the TCA-Soluble

and TCA-Insoluble Fractions—Incorporations of S^{35} into the both TCA-soluble and TCA-insoluble fractions of the parent sensitive strain were inhibited significantly by the presence of 1 μg. of kanamycin per ml. (Table II). Such inhibitions occurred markedly also in the presence of 10 μg. of streptomycin per ml. as well as 5 μg. of viomycin per ml. Inhibition of protein synthetis by streptomycin and that by viomycin were recently reported by Erdös and Ullmann (*6*) and Tsukamura (*7*), respectively.

TABLE II

Distribution of Radioactivity from Incorporated S^{35} in Cellular Fractions of Parent Sensitive Strain

Reaction System[1)]	Time of Incubation	Radioactivity as c.p.m. per 1 μg. of DNA	
		TCA-Soluble Fraction	TCA-Insoluble Fraction
Control	1 Hour	12.4	9.22
KM 1 μg./ml.		8.39	4.86
SM 10 μg./ml.		3.46	5.53
VM 5 μg./ml.		5.99	3.50
Control	3 Hours	16.3	17.4
KM 1 μg./ml.		5.78	9.11
SM 10 μg-/ml.		3.15	3.67
VM 5 μg./ml.		4.40	4.87
Control	5 Hours	21.8	19.6
KM 1 μg./ml.		15.1	9.56
SM 10 μg./ml.		5.18	2.40
VM 5 μg./ml.		7.54	2.56

1) Reaction system (4.0 ml.): Cells suspended in 0.033 *M* phosphate buffer, pH 7.1, 25 mg./ml.; S^{35}-sulfate, 10 μc/ml.; No drug, Kanamycin sulfate (KM) 1 μg./ml., dihydrostreptmycin sulfate (SM) 10 μg./ml., or viomycin sulfate (VM) 5 μg./ml. (in final concentrations)

The incorporation of S^{35} into the TCA-insoluble fraction was inhibited much more markedly in 0.05 *M* sodium citrate solutions than in 0.03 *M* phosphate buffer solutions (Data are not shown). This finding was compatible with the fact that antimycobacterial action of kanamycin decreased by increasing concentrations of phosphate (*8*).

The concentration of kanamycin (1 μg./ml.) used in these experiments inhibited almost completely the growth of the parent sensitive strain after a lag period of a few multiplications. It acted, however, only partially bactericidal. As shown in table III, loss of viability occurred only in a portion of the total bacterial population after a 3 hour incubation in the test concentration.

Incorporations of Radioative Phosphorus (P^{32}) and Radioactive Sulfur (S^{35}) into the Cellular Fractions of the Kanamycin-Resistant Strain in the Presence and Absence

of Kanamycin—Incorporations of P^{32} and S^{35} into different cellular fractions of the kanamycin-resistant strain were not inhibited by the presence of 1 μg. of kanamycin per ml. (Tables IV and V). In addition, it was observed that, in the absence of kanamycin, the incorporations of the isotopes into cellular fractions occurred more rapidly in the kanamycin-resistant strain than in the parent sensitive strain (Compare Tables I and II with Tables IV and V). This finding suggests that the turnover rate was higher in the kanamycin-resistant strain, because the amount of DNA remained constant throughout the study.

TABLE III

Loss of Viability after the Contact with Kanamycin for 3 Hours

Kanamycin Concentration	Number of Viable Cell Units per mg., Wet Weight of Cells[1]
0 μg./ml.	2.03×10^6/mg. (100.0%)
1	1.32×10^6/mg. (65.0%)
10	7.60×10^5/mg. (37.4%)

1) Determined by usual plate count method after washing cells three times with saline.

Reaction system consisted of washed cells, 0.70 to 0.76 mg./ml.; 0.033 M phosphate buffer, pH 7.1; and kanamycin, 0, 1, or 10 μg./ml. (in final concentrations).

TABLE IV

Distribution of Radioactivity from Incorporated P^{32} in Cellular Fractions of Kanamycin-Resistant Strain

Reaction System[1]	Time of Incubation	Radioactivity as c.p.m. per 1 μg. of DNA — Fraction: Acid-Soluble	Phosphatide	Nucleic Acid	Protein
Control	1 Hour	22.6	2.48	40.0	2.10
KM 1 μg./ml.		21.6	4.12	38.0	2.24
Control	2 Hours	21.7	2.77	57.3	5.10
KM 1 μg./ml.		23.4	2.47	61.1	2.90
Control	3 Hours	17.3	2.40	50.8	5.60
KM 1 μg./ml.		28.8	4.20	50.3	3.93
Control	5 Hours	22.5	2.25	69.1	10.0
KM 1 μg./ml.		24.6	2.92	52.8	9.30

1) Reaction system (4.0 ml): Cells suspended in phosphorus deficient medium, 18 mg./ml.; P^{32}-orthophosphate, 1.5 μc/ml.; Kanamycin sulphate (KM), 0 or 1 μg./ml. (in final concentrations)

The incorporations of the isotopes were as significantly inhibited by the presence of 10 μg. of streptomycin per ml. or 5 μg. of viomycin per ml. as in the parent sensitive strain (Tables II and V).

TABLE V

Distribution of Radioactivity from Incorporated S^{35} in Cellular Fractions of Kanamycin-Resistant Strain

Reaction System[1)]	Time of Incubation	Radioactivity as c.p.m. per 1 μg. of DNA	
		TCA-Soluble Fraction	TCA-Insoluble Fraction
Control	1 Hour	13.9	23.3
KM 1 μg./ml.		13.9	19.6
SM 10 μg./ml.		5.68	6.40
VM 5 μg./ml.		9.00	10.6
Control	3 Hours	26.0	78.2
KM 1 μg./ml.		22.5	51.0
SM 10 μg./ml.		9.53	7.64
VM 5 μg./ml.		14.3	12.4
Control	5 Hours	26.0	49.8
KM 1 μg./ml.		32.2	60.1
SM 10 μg./ml.		6.71	11.9
VM 5 μg./ml.		16.7	22.0

1) Reaction system (4.0 ml.): Cells suspended in 0.033 *M* phosphate buffer, pH 7.1, 25 mg./ml.; S^{35}-sulfate, 10 μc/ml.; No drug, kanamycin sulfate (KM) 1 μg./ml., dihydrostreptomycin sulfate (SM) 10 μg./ml., or viomycin sulfate (VM) 5 μg./ml. (in final concentrations)

TABLE VI

The RNA : DNA Ratios Determined on 8-Day-Old Cultures of Both Kanamycin-Sensitive and -Resistant Strains

Strain	RNA : DNA[1)]
Parent Sensitive	6.39±0.684
Kanamycin-Resistant	12.32±0.965

1) (Mean of eight replicates)±(Standard deviation)

In addition, it was found that the RNA : DNA ratio was higher in the kanamycin-resistant strain than in the kanamycin-sensitive strain (Table VI). This finding may be related to the rapid incorporations of isotopes into the nucleic acid and protein fractions of the kanamycin-resistant strain.

DISCUSSION

The results show that the incorporation of P^{32} into the nucleic acid and protein fractions of the parent sensitive strain are inhibited by the presence of only 1 μg. of kanamycin per ml., and the incorporations of S^{35} into the TCA-insoluble and TCA-insoluble (protein) fractions of the parent sensitive strain are also inhibited by the same concentration of the drug. The concentration of the drug used was as causing only a sight loss of viability.

It had been confirmed that, under the conditions tested, the oxygen uptake of the test organism by the conventional Warburg method is not affected even in the presence of 100 μg. of kanamycin per ml., which is 100 times higher than the concentration of the drug used in the present study (without substrate and with glucose, pyruvate, acetate and succinate added in a concentration of 0.02 *M*). Therefore, it appears to be apparent that the inhibition of the incorporations of the isotopes is not due to the inhibition of respiration (Author's unpublished data).

On the contrary, it was demonstrated that, in the kanamycin-resistant strain, the incorporations of both isotopes, P^{32} and S^{35}, were not inhibited in the presence of the same concentration of the dug.

The results above obtained indicate that the incorporation of P^{32} into the nucleic acid and protein fractions and the incorporation of S^{35} into the protein fraction of *M. avium* are inhibited in the presence of kanamycin (1 μg./ml.). This finding seems to explain the data obtained by Aoki *et al.* (*2*), who showed the inhibition of the adaptive oxidation of benzoic acid, niacin and malonic acid by *Mycobacterium avium* in the presence of kanamycin (but much higher concentration, 100 μg./ml.), because the adaptive oxidation is closely related to the function of RNA (*9*, *10*). It is noteworthy that the kanamycin-resistant strain has a higher ratio of RNA : DNA and, furthermore, incorporations of the isotopes occur more rapidly in the kanamycin-resistant strain.

SUMMARY

1. Kanamycin inhibits the incorporations of P^{32} into the nucleic acid and protein fractions and the incorporations of S^{35} into the TCA-soluble and protein (TCA-insoluble) fractions of the parent sensitive strain of *Mycobacterium avium*, strain Jucho.

2. The incorporations of P^{32} and S^{35} into cellular fractions of the kanamycin-resistant strain are not inhibited by kanamycin. Kanamycin-resistant strain has rapid incorporaiions of P^{32} and S^{35} into the nucleic acid and protein fractions. The RNA : DNA ratio is higher in the kanamycin-resistant strain than in the parent sensitive strain.

REFERENCES

(*1*) Tsukamura, M., and Tsukamura, S., *J. Biochem.*, **46**, 1193 (1959)

(*2*) Aoki, T., Hayashi, A., and Ito, F., *J. Antibiotics, A*, **12**, 98 (1959)
(*3*) Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945)
(*4*) Dische, Z., *Mikrochemie*, **8**, 4 (1930)
(*5*) Kerr, S. E., and Seraidarian, K., *J. Biol. Chem.*, **159**,211 (1945)
(*6*) Erdös, T., and Ullmann, A., *Nature*, **183**, 618 (1959)
(*7*) Tsukamura, M., *J. Biochem.*, **47**, 685 (1960)
(*8*) Tsukamura, M., *Medicine and Biology* (in Japanese), **51**, 140 (1959)
(*9*) Caspersson, T., *Symposia Soc. Exptl. Biol.*, **1**, 127, (1947)
(*10*) Spiegelman, S., *Symposia Soc. Exptl. Biol.*, **2**, 286 (1948)

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STUDIES ON THE OXIDATIVE DECOMPOSITION OF UROCANIC ACID

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In the previous report (*1*) evidences were presented that oxygen uptake during urocanic acid decomposition by liver and bacterial enzymes, was due to the oxidation of imidazolonepropionic acid, the product of the action of urocanase, and that hydrogen peroxide was involved in this oxidation. The initiation of the oxidation of imidazolonepropionic acid by hydrogen peroxide suggested that the reaction was of a peroxidatic character. This paper reports on the peroxidatic nature of the oxidation reaction catalyzed by a urocanase preparation obtained from cat liver. It will be shown that copper is involved in the reaction.

EXPERIMENTAL

Materials and Methods

1. Urocanic acid was prepared enzymatically from L-histidine according to the procedure of Mehler, Tabor and Hayaishi (*2*) with a partial modification. Urocanic acid was determined spectrophotometrically by its ultra-violet absorption at 277 mμ. The molecular extinction coefficient of urocanic acid at neutral pH was 18,800.
2. α-Ketoglutaric acid amide was determined as its 2,4-dinitrophenylhydrazone by the method described previously (*1*).
3. Horse-radish peroxidase was purified by ammonium sulfate precipitation by the method of Kenten and Mann (*3*).
4. A lyophilized glucose oxidase preparation was obtained from Nagase Industry Co., Amagasaki, Japan.
5. Beef liver catalase was prepared by the method of Sarker and Sumner (*4*).

RESULTS AND DISCUSSION

Cat liver urocanase was partially purified by acid and ammonium sulfate precipitation, calcium phosphate gel adsorption, and heat treatment (*1*). Until heat treatment, the urocanase preparation contained an enzyme system which converted urocanic acid to formamidinoglutaric acid. Heat treatment of calcium phosphate gel fraction (Fraction CG) at 63° for one minute in the presence of urocanate (*5*) (0.04 *M*; final concentration) destroyed the imidazolone hydrase of Feinberg and Greenberg (*6*), an

enzyme forming formamidinoglutaric acid from imidazolonepropionic acid. When this heat treated urocanase preparation (Fraction H) was incubated with urocanic acid, oxygen uptake was observed and α-ketoglutaric acid amide and other oxidation products were formed. In the following experiments, α-ketoglutaric acid amide formation was used as a measure of oxidation for the elucidation of the oxidation mechanism.

During the study of the oxygen consumption by this enzyme preparation, it was fouud that oxygen uptake was completely inhibited by crystalline beef liver catalase, and that the lag in oxygen uptake could be eliminated by a small amount of added hydrogen peroxide (*1*). As shown in Table I, the inhibition of oxygen uptake by catalase accounted for the inhibition of α-ketoglutaric acid amide formation.

TABLE I

Effect of Catalase on Oxygen Uptake and KGAA Formation*

Catalase added	Total O_2 uptake	KGAA Formed
mg.	μl.	μM
0	56.6	1.37
1.6	21.2	0.93
3.2	12.5	0.59

100 μM of phosphate buffer, pH 7.4, 6 μM of urocanic acid, 6 mg. of Fraction H protein and beef liver catalase as indicated, in a total volume of 3.0 ml., were incubated in Warburg vessels at 37° for 120 minutes.

TABLE II

KGAA Formation by Fraction CG in Presence of an H_2O_2-generating System and Horse-radish Peroxidase

System		KGAA formed
		μM
Complete		0.30
,,	—UCA	0.00
,,	—glucose and glucose oxidase	0.01
,,	—horse-radish peroxidase	0.01

Complete system contained, in a total volume of 2.0 ml, 100 μM of phosphate buffer, pH 7.4, 2 μM of urocanic acid, 0.3 mg. of Fraction CG, 4.0 mg. of glucose, 0.4 mg. of glucose oxidase preparation and 0.2 ml. of horse-radish peroxidase. Incubated at 37° for 60 minutes.

As mentioned above, when Fraction CG was incubated with urocanic acid formamidinoglutaric acid was formed stoichiometrically from urocanic

* The following abbreviations are used: KGAA=α-ketoglutaric acid amide; UCA=urocanic acid; DDC=sodium diethyldithiocarbamate; DEAE-cellulose=diethylaminoethylcellulose.

acid and no oxygen was consumed. However, when Fraction CG was incubated with urocanic acid and horse-radish peroxidase and an H_2O_2-generating system, α-ketoglutaric acid amide and other oxidation products were produced. This indicates that imidazolonepropionic acid formed by the action of urocanase acts as a substrate for peroxidase.

Peroxidatic Nature of Oxidation Reaction—As mentioned above, the initiation of oxidation by hydrogen peroxide suggested two possibilities for the oxidation mechanism. One is due to the oxidase action of peroxidase. Theorell and Swedin (*7*), Chance (*8*), Galston, Bonner, and Baker (*9*), Ray (*10*), and Yamazaki (*11*) reported the reactions of this type using dihydroxyfumaric acid, indoleacetic acid and triose reductone as peroxidase substrates. It must be noted that these substances are easily oxidizable and this is also the case with imidazolonepropionic acid in our system (*1*, *6*). Another possibility is the activation of an oxidizing enzyme by hydrogen peroxide. This type has been reported by Tanaka and Knox in the tryptophan pyrrolase reaction (*12*). The latter possibility was disproved by the experiment presented in Table III. Fraction H was incubated with urocanic acid under aerobic and anaerobic conditions.

TABLE III

Requirement for H_2O_2 for Formation of KGAA in Absence of Oxygen

Expt. No.	H_2O_2 added	UCA disappeared	KGAA formed
	μM	μM	μM
1[a]	0	1.0	0.28
2	0	1.0	0.04
3	20	1.0	0.13
4	40	1.0	0.31

a) Aerobic

100 μM of phosphate buffer, pH 7.4, 1.0 μM of urocanic acid and 0.12 mg. of Fraction H, in a total volume of 2.0 ml., were incubated under N_2 in Thunberg tubes for 60 minutes at 37°.

Aerobically α-ketoglutaric acid amide and a carbamyl derivatives were formed. However, in the absence of oxygen, the main product was formyl-DL-isoglutamine, which was formed non-enzymatically from imidazolonepropionic acid (*1*). As shown in Table III, when Fraction H was incubated with urocanic acid under N_2 with added hydrogen peroxide, α-ketoglutaric acid amide was detected in the incubation mixture. Fraction H protein used had no catalase activity. Addition of hydrogen peroxide during aerobic incubation did not increase the rate of α-ketoglutaric acid amide formation.

Effect of Cyanide on Formation of α-Ketoglutaric Acid Amide—It was very difficult to show the effect of cyanide on α-ketoglutaric acid amide forma-

tion, since urocanase which gives rise to imidazolonepropionic acid, the substrate for the oxidation, is considerably inhibited by cyanide (*1*, *6*, *13*). However, it was found that the α-ketoglutaric acid amide forming system was more sensitive than urocanase to cyanide. Therefore, to demonstrate the effect of cyanide on α-ketoglutaric acid amide formation clearly, the ratio of 'α-ketoglutaric acid amide formed/urocanic acid disappeared' was measured at various concentrations of cyanide. The result is shown in Fig. 1. Azide did not inhibit either the urocanase or the α-ketoglutaric acid amide forming system.

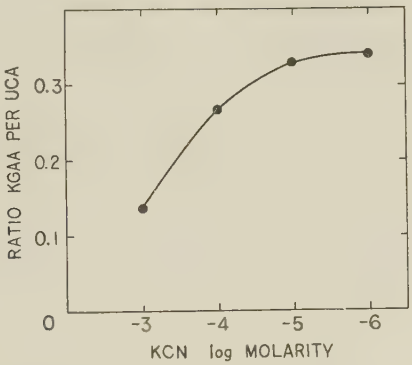

FIG. 1. Effect of cyanide on KGAA formation.

100 μM of phosphate buffer, pH 7.4, 0.12 mg. of Fraction H, and potassium cyanide as indicated were preincubated for 15 minutes at 37°. Reaction was started by the addition of 1 μM of urocanic acid. Total volume, 2.0 ml. Incubation for 120 minutes at 37°.

TABLE IV

Inhibitory Effect of DDC on KGAA Formation

Final concentration of DDC	KGAA formed	Per cent inhibition
M	μM	%
0	0.58	0
1×10^{-4}	0.51	12.6
3×10^{-4}	0.34	41.8

100 μM of phosphate buffer, pH 6.8, 0.12 mg. of Fraction H, and DDC, in a volume of 1.9 ml., were preincubated for 15 minutes. The reaction was started by the addition of 2.0 μM (0.1 ml.) of urocanic acid. Incubated at 37° for 60 minutes.

Higher concentrations of DDC gave irregular results possibly due to destruction of DDC.

Absence of Iron Porphyrin Protein in Urocanase Preparation—The inhibitory action of cyanide on α-ketoglutaric acid amide formation suggested the

participation of some heavy metal in the reaction. This and the peroxidatic nature of the reaction strongly suggested the presence of an iron porphyrin protein in the preparation acting as a peroxidase.

The absorption spectrum of Fraction H protein, however, had no iron porphyrin-like absorption maxima. Moreover, no pyridine hemochromogen was formed when Fraction H was mixed with pyridine-NaOH.

No peroxidase activity of Fraction H protein could be demonstrated using reduced indophenol dye or guaiacol as a hydrogen donor.

Participation of Copper in Oxidation Reaction—α-Ketoglutaric acid amide formation by Fraction H protein was inhibited by sodium diethyldithiocarbamate. Urocanase activity was not affected by diethyldithiocarbamate. As the addition of sodium diethyldithiocarbamate at higher concentrations

TABLE V

Inhibition of KGAA Formation by DDC-treated Enzyme and Reversal of Inhibition by Copper

Expt. No.	$CuSO_4$: final concen.	UCA disappeared	KGAA formed	Ratio KGAA/UCA
	M	μM	μM	
1[a]	0	1.00	0.30	0.30
2	0	1.00	0.06	0.06
3	1×10^{-6}	1.00	0.10	0.10
4	1×10^{-5}	0.92[b]	0.24	0.26

a) In Experiment 1, Fraction H protein not treated with DDC was used.

b) Higher concentrations of Cu^{++} inhibited the urocanase activity.

100 μM of phosphate buffer, pH 7.4, and 0.95 mg. of DDC-treated Fraction H, with or without $CuSO_4$, in a volume of 1.9 ml., were preincubated for 15 minutes at 37°. The reaction was started by the addition of 1.0 μM (0.1 ml.) of urocanic acid. Incubated for 90 minutes at 37°.

gave irregular results, the following experiment was undertaken. Diethyldithiocarbamate at a final concentration of 1×10^{-3} *M* was added to Fraction H. After standing for 30 minutes at 2°, the mixture was dialyzed against 250 volumes of distilled water for three hours with three changes of water. This treatment completely removed the inhibitor.

There was no significant oxygen uptake or α-ketoglutaric acid amide formation with diethyldithiocarbamate-treated enzyme during urocanic acid decomposition. This inhibition of oxidation was reversed by the addition of a low concentration of $CuSO_4$. Addition of $CuSO_4$ to the incubation mixture to a final concentration of 1×10^{-5} *M*, almost completely reversed the inhibition caused by sodium diethyldithiocarbamate. Since about 1 mg. of Fraction H protein was used in this experiment, the concentrations of protein and of $CuSO_4$ in the incubation mixture were of the same order of magnitude.

Fe^{++}, Fe^{+++}, Mn^{++} and Ni^{++} did not reverse the inhibition.

When fraction H protein not treated with diethyldithiocarbamate and dialyzed against distilled water was used, there was no decrease in the ability to form α-ketoglutaric acid amide from urocanic acid. It is, therefore, assumed that the reaction is catalyzed not by free but by bound copper. The nature of the bound copper is unknown. It is not clear whether the peroxidation of imidazolonepropionic acid is catalyzed by a specific or non-specific copper-protein, or by the urocanase to which the metal is in some way bound. However, the elucidation of this mechanism is very difficult because imidazolonepropionic acid is not isolated.

Similar results were obtained for the oxidation of imidazolonepropionic acid by a bacterial urocanase preparation. It was reported that the urocanase of *Pseudomonas aeruginosa* when treated as described above, showed no oxygen uptake (*1*).

From the above data, it is seen that α-ketoglutaric acid amide is formed from imidazolonepropionic acid by a peroxidatic reaction and that copper is involved in this reaction.

Attempts to separate the urocanase activity from the peroxidase active fraction by column chromatography on DEAE-cellulose, by adsorption on calcium phosphate gel or alumina C_γ gel were without success.

SUMMARY

1. α-Ketoglutaric acid amide, one of the oxidation products of urocanic acid, is formed by a peroxidatic reaction.

2. Since α-ketoglutaric acid amide formation is inhibited by potassium cyanide and by sodium diethyldithiocarbamate, and since the inhibition by the latter compound can be reversed by the addition of a low concentration of copper, it is assumed that the peroxidatic reaction is catalyzed by a Cu-protein.

3. Iron porphyrin protein does not seem to be involved in this reaction.

REFERENCES

(*1*) Satani, H., Kakiuchi, S., Fujioka, M., Nakahara, I., Okada, N., Sakamoto, Y., and Ichihara, K., *J. Biochem.*, **48**, 121 (1960)

(*2*) Mehler, A. H., Tabor, H., and Hayaishi, O., "Biochemical Preparations", Westerfield, W. W. Editor-in-Chief, John Wiley and Sons, Inc., New York, Vol. **4**, 1955, p. 50.

(*3*) Kenten, R. H., and Mann, P. J. G., *Biochem. J.*, **57**, 347, (1954)

(*4*) Sumner, J. B., and Dounce, A. L., "Methods in Enzymology", Colowick, S.P., and Kaplan, N. O., Eds., Academic Press, Inc., New York, Vol. **II**, 1955. p. 777.

(*5*) Suda, M., and Shimomura, H., *J. Japan. Biochem. Soc.*, **28**, 319 (1956)

(*6*) Feinberg, R. H., and Greenberg, D. M., *J. Biol. Chem.*, **234**, 2670 (1959)

(*7*) Theorell, H., and Swedin, B., *Naturwiss.*, **27**, 95 (1939)

(*8*) Chance, B., *J. Biol. Chem.*, **197**, 577 (1952)

(*9*) Galston, A. W., Bonner, J., and Baker, R. S., *Arch. Biochem. Biophys.*, **42**, 456 (1953)

(*10*) Ray, P. M., *Arch. Biochem. Biophys.*, **64**, 193 (1956)
(*11*) Yamazaki, I., *J. Biochem.*, **44**, 425 (1957)
(*12*) Tanaka, T., and Knox, W. E., *J. Biol. Chem.*, **234**, 1162 (1959)
(*13*) Miller, A., and Wealsch, H., *J. Biol. Chem.*, **228**, 365 (1957)

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CONDENSATION PRODUCTS OF ETHYLENEDIAMINE WITH CATECHOL DERIVATIVES

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It had been reported by Natelson *et al.* (*1*) and Weil-Malherbe *et al.* (*2*) that adrenaline and noradrenaline were converted quantitatively into strongly fluorescent and stable products by their reaction with ethylenediamine, and Weil-Malherbe *et al.* (*3*) devised the fluorimetric separative determination of adrenaline and noradrenaline by use of two kinds of secondary filters, based on the difference of the fluorescence spectra of the condensation products of both catechol amines. They also assumed that the reaction proceeds by an oxidation of adrenaline to adrenochrome and the condensate is derived by simple condensation of adrenochrome with ethylenediamine. Thereafter, it had been commonly assumed that each catechol derivative forms a single fluorescent condensate respectively.

This reaction, however, has been found to be more complicated and to yield several fluorescent side products besides the main condensate (*4–8*). Although the structure of the main condensation product of ethylenediamine with adrenaline had been reported to be dihydro-3-hydroxy-1-methylpyrrolo-[4:5-g]-quinoxaline (*7*), and that of catechol to be 1:2:3:4-tetrahydro-1:4:5:8-tetraaza-anthracene, the other main condensates have been unknown. Moreover, precise properties of these main condensates have not been reported.

Considering the importance of this reaction in the fluorimetric determination of catechol amines, the precise studies of the condensates of various catechol derivatives might be necessary. Thus, the separation of the condensation products of various catechol derivatives by means of paper chromatography and paper electrophoresis and the examination of the physico-chemical properties of their main condensates were studied. The preliminary notes had appeared (*8*, *9*).

MATERIALS

The following preparations were used: L-adrenaline, a product of Merck Co.; DL-noradrenaline, a product of Eastman Chemical Incorp.; 3,4-dihydroxy-DL-mandelic acid, kindly supplied by Dr. M. D. Armstrong, the Fels Research Institute, Ohio; dopamine, kindly synthesized by Dr. T. Kametani, Tohoku University, Sendai; DL-dopa and catechol, commercial products.

When the sample of L-adrenaline was tested by paper chromatography using phenol containing 15 per cent (*v/w*) 0.1 *N* HCl as solvent (*10*) and coloured by potassium ferricyanide (*11*), only one spot appeared. Only one peak was also observed by IRC-50 column chromatography (*12*). Therefore, the presence of noradrenaline or of other catechol derivatives could be excluded. The purity of the other catechol derivatives was also checked by paper chromatography and by IRC-50 column chromatography, as described above. Each catechol derivative was dissolved into 0.01 *N* HCl. Ethylenediamine, or ethylenediamine monohydrate was redistilled. Ethylenediamine dihydrochloride solution (2 *M*) was prepared as reported by Weil-Malherbe *et al.* (*2*).

METHODS

Formation of the Condensation Products of Ethylenediamine with Catechol Derivatives—The procedure reported by Weil-Malherbe *et al.* (*2*) was re-examined. The essential feature of this method was as follows: the condensation is carried out by heating 10 ml. of the solution of catechol derivative with a mixture of 0.7 ml. ethylenediamine and 0.5 ml. ethylenediamine dihydrochloride solution (2 *M*) (pH of the mixture becomes 10.4) at 50° for 20 min.

The maximum fluorescence was obtained at pH 10.4 (Table I).

TABLE I

Effect of pH of the Reaction Mixture on the Condensation of Adrenaline with Ethylenediamine

pH[1)]	9.4	9.8	10.0	10.2	10.4	10.8
Fluorescence intensity (%)	65.0	75.0	82.0	98.0	100.0	95.0

1) pH of the reaction mixture containing adrenaline and ethylenediamine was adjusted by the addition of HCl, and determined by glass-electrode pH meter. The reaction mixture was heated at 50° for 20 min., and then extracted with *iso*-butanol. The fluorescence intensity of *iso*-butanol layer was determined by a fluorimeter designed by Yagi *et al.* (*13*).

The volume of the mixture of ethylenediamine base and 2 *M* dihydrochloride used in the reaction by Weil-Malherbe *et al.* (*2*) was found to be optimum. Although their condition of the reaction, at 50° for 20 min., was enough for adrenaline, this condition was not sufficient for the other catechol derivatives. The stronger fluorescence could be obtained by the lower temperature and the longer time reaction, as already reported by Erne *et al.* (*14*). Great loss of the fluorescence was observed with noradrenaline, 3,4-dihydroxymandelic acid, or catechol, when the reaction was carried out in day light, as already reported with noradrenaline by Goldfien *et al.* (*15*). In the case of dopa, the fluorescence colour of the reaction mixture changed during the heating and the fluorescence spectrum became constant after 2 hours' heating at 50° (*16*).

After these preliminary experiments, the modifications were applied to the method as follows: To 5 μg. of each catechol derivative in 10 ml. of water, 0.5 ml. of 2 *M* ethylenediamine dihydrochloride and 0.7 ml. of anhydrous ethylenediamine base (or 1 ml. of ethylenediamine hydrate) was added and heated in a water bath at 50° for 2 hours in

a dark. After cooling, the mixture was saturated with solid sodium chloride (4 g.) and extracted with 6 ml. of *iso*-butanol for 4 min. The butanol layer was separated by centrifugation.

In order to concentrate the fluorescent derivatives, 5 ml. of the *iso*-butanol extract were condensed under reduced pressure at about 30° in a dark and redissolved with a small amount of water. In the case of dopa, the reaction mixture was heated at 50° for 2 hours, and the *iso*-butanol extraction was omitted, because dopa forms a derivative which could not be extracted by *iso*-butanol from the alkaline solution (*2*). The reaction mixture was condensed to the adequate concentration for paper chromatography or for paper electrophoresis.

Separation of the Condensation Products by Paper Chromatography—The strips of Whatman No. 1 filter paper (2.5×40 cm.) were washed by ethylenediamine tetraacetate as described by Weil-Malherbe *et al.* (*17*): the strips were steeped in 0.5 *N* HCl (three changes) for 24 hours and washed in several changes of distilled water; they were then immersed in a solution of 0.1 per cent ethylenediamine tetraacetate for 24 hours, washed and air-dried. This procedure can be eliminated, when the solvents of group I were used. In this case, the condensate derived from ethylenediamine and filter paper moves to the front of the solvent and does not disturb the analysis as described in the results.

The following solvents were used:

Group I (1) 5% $Na_2HPO_4 \cdot 12H_2O$
(2) *n*-butanol-saturated phosphate buffer (pH 5.8, 0.05 *M*) (*6*)

Group II (3) methanol/conc. NH_4OH (9/1, *v/v*) (*18*)
(4) *n*-butanol/acetic acid (100/15, *v/v*) saturated with water (*6*)
(5) the upper layer of *n*-butanol/ethanol/5% $Na_2HPO_4 \cdot 12H_2O$ (50/25/30, *v/v*) (*19*)
(6) the upper layer of *n*-butanol/methanol/5% $Na_2HPO_4 \cdot 12H_2O$ (60/15/30, *v/v*) (*19*)
(7) the upper layer of *n*-propanol/*n*-butanol/5% $Na_2HPO_4 \cdot 12H_2O$ (50/15/30, *v/v*) (*19*)
(8) *n*-butanol/acetone/*iso*-pentanol/water saturated with boric acid (50/20/5/30, *v/v*) (*19*)
(9) *n*-butanol/acetone/*iso*-propanol/water saturated with boric acid (50/15/15/30, *v/v*) (*19*)

The aqueous solution containing the condensation products corresponding to about 0.3 μg. of each catechol derivative was placed on a filter paper strip of Whatman No. 1. The paper strip was developed in a dark at about 20° either by ascending (in the case of the solvents of group I) or by descending method (in the case of the solvents of group II). The condensation products on the filter paper were examined under ultraviolet light of 365 mμ.

Separation of the Condensation Products by Paper Electrophoresis—The aqueous solution containing the condensation product corresponding to about 1 μg. of each catechol derivative was placed on the anode side of a paper strip (Whatman No. 1, 2×32 cm.) moistened with phosphate buffer (pH 8.0, 0.05 *M*), and 1 mA/cm. (300–400 volts) of constant current was applied in a dark. After electrophoresis, the paper strip was examined under ultraviolet light.

Absorption Spectrum and Fluorescence Spectrum of the Main Condensate of Each Catechol Derivative with Ethylenediamine—Larger amount of each catechol derivative was condensed with ethylenediamine as described above, and the main condensate was separated from the reaction mixture by paper chromatography using 5 per cent $Na_2HPO_4 \cdot 12H_2O$ as mobile phase. The part of the paper strip containing the main condensate was detected under ultraviolet light and the condensate was extracted with water at 50° for 1 hour. Absorption

spectrum of this aqueous extract was measured by using the aqueous extract of the paper strip without catechol derivative as blank.

The condensate was extracted with *iso*-butanol from the aqueous solution, and its fluorescence spectrum was measured. The main condensate of dopamine could be extracted with *iso*-butanol after the aqueous extract was made alkaline with NaOH. For the estimation of the fluorescence spectrum of the main condensate of dopa, the reaction mixture after 2 hours' heating at 50° was used. The fluorescence spectrum was measured by the fluorospectrophotometer designed by Yagi *et al.* (*20*).

pH-Fluorescence Curve of the Main Condensate of Each Catechol Derivative with Ethylenediamine—Each main condensate was separated on the filter paper and was extracted with water as described above. This sample was dissolved in the buffers of different pH's. The buffer was a mixture of equivolume of 0.1 *M* glycine, 0.1 *M* Na_2HPO_4, 0.1 *M* KH_2PO_4, and the pH was adjusted by adding 0.1 *N* NaOH or 0.1 *N* HCl. For below pH 1, or above pH 13, however, the aqueous solution of HCl or NaOH was used. The relative fluorescence intensity of the solution at indicated pH, excited by ultraviolet light (365 mμ), was measured at about 20° by a fluorimeter designed by Yagi *et al.* (*13*).

RESULTS

I. Separation of the Condensation Products by Paper Chromatography

Adrenaline Condensates—When the sample containing the condensation products corresponding to about 0.3 μg. of adrenaline was chromatographed by using *n*-butanol/ethanol/5 per cent $Na_2HPO_4 \cdot 12H_2O$ (50/25/30, *v/v*) as solvent, the following three fluorescent spots appeared: weak violet fluorescent spot at R_f 0.32, yellow fluorescent spot at R_f 0.48, and bluish-green fluorescent spot at R_f 0.71.

The violet fluorescent spot at R_f 0.32 was considered to be derived from the substance contained in the filter paper. The reasons are as follows: (i) this spot could also be observed in the case of the blank test without adrenaline; (ii) ethylenediamine, when applied on the filter paper, formed a violet fluorescent spot which is easily extractable with hot water and has the same fluorescence spectrum as that of the extract from the spot at R_f 0.32 (Fig. 1). So, this violet fluorescent spot was attributed to the substance produced by the reaction of ethylenediamine dissolved in *iso*-butanol with the substance contained in filter paper. When the solvents of group I were used, the violet fluorescent spot derived from ethylenediamine with filter paper was observed at the solvent front.

The strongest yellow fluorescent spot, at R_f 0.48 was the main product which is specific for adrenaline.

The bluish-green fluorescent spot at R_f 0.71 was considered to be identical with the main spot of noradrenaline derivative as described below.

Noradrenaline Condensates—When the sample, containing the condensation products corresponding to about 0.3 μg. of noradrenaline, was chromatographed by *n*-butanol/ethanol/5 per cent $Na_2HPO_4 \cdot 12H_2O$ (50/25/30, *v/v*) as solvent, three spots were observed as follows: yellow fluorescent spot at R_f 0.04; violet fluorescent spot at R_f 0.32; and bluish-green fluorescent spot at R_f 0.71.

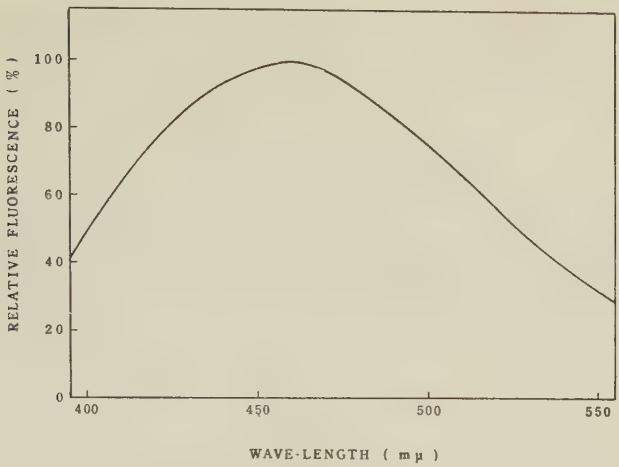

FIG. 1. Fluorescence spectrum of an aqueous solution of a substance derived from the reaction of ethylenediamine with filter paper. The ordinate shows the relative intensity of the fluorescence.

TABLE II

R_f Values of the Main Condensates of Catechol Derivatives with Ethylenediamine

Catechols	adrenaline		noradrenaline	dopa	dopamine	dihydroxymandelic acid	catechol
	main product	side product					
Colour of fluorescence	yellow	bluish-green	bluish-green	greenish-yellow	yellowish-green	bluish-green	bluish-green
Solvent 1	0.37	0.09	0.09	0.29	0.27	0.09	0.09
2	0.52	0.20	0.20	0.44	0.33	0.20	0.20
3	0.50	0.57	0.57	0.07	0.09	0.57	0.57
4	0.37	0.64	0.64	0.01	0.01	0.64	0.64
5	0.48	0.71	0.71	0.02	0.05	0.71	0.71
6	0.37	0.73	0.73	—	—	—	—
7	0.46	0.77	0.77	—	—	—	—
8	0.50	—	0.77	—	—	—	—
9	0.46	—	0.76	—	—	—	—

Solvent 1: 5% $Na_2HPO_4 \cdot 12H_2O$
2: *n*-butanol-saturated phosphate buffer (pH 5.8, 0.05 *M*)
3: methanol/conc. NH_4OH (9/1, *v/v*)
4: *n*-butanol/acetic acid (100/15, *v/v*) saturated with water
5: the upper layer of *n*-butanol/ethanol/5% $Na_2HPO_4 \cdot 12H_2O$ (50/25/30, *v/v*)
6: the upper layer of *n*-butanol/methanol/5% $Na_2HPO_4 \cdot 12H_2O$ (60/15/30, *v/v*)
7: the upper layer of *n*-propanol/*n*-butanol/5% $Na_2HPO_4 \cdot 12H_2O$ (50/15/30, *v/v*)
8: *n*-butanol/acetone/*iso*-pentanol/water saturated with boric acid (50/20/5/30, *v/v*)
9: *n*-butanol/acetone/*iso*-propanol/water saturated with boric acid (50/15/15/30, *v/v*)

The strongest bluish-green fluorescent spot at R_f 0.71 was the main condensation product which was specific for noradrenaline.

Condensates of the Other Catechol Derivatives—The *iso*-butanol extract of condensates of 3,4-dihydroxymandelic acid or dopamine gave one markedly fluorescent spot and other lesser fluorescent spot, whereas that of catechol gave only one spot fluoresced bluish-green.

In the case of dopa, however, the *iso*-butanol extract of its condensate showed no fluorescent spot in paper chromatography, though the reaction mixture showed marked greenish-yellow fluorescent spot having the R_f value of 0.02, when developed by *n*-butanol/ethanol/5 per cent $Na_2HPO_4 \cdot 12H_2O$ (50/25/30, *v/v*). This fact showed that dopa formed its main condensate which could not be extracted by *iso*-butanol from the alkaline solution, or only to a slight extent, as reported by Weil-Malherbe *et al.* (*2*).

The R_f value of the main condensate of each catechol derivative was shown in Table II. The main condensate of adrenaline, noradrenaline, dopamine and dopa had the different R_f values, whereas those of noradrenaline, dihydroxymandelic acid and catechol and the side product of adrenaline had the same R_f value.

II. Separation of the Condensation Products by Paper Electrophoresis

As shown in Table III, the main condensates of adrenaline, noradrenaline, dopamine, and dopa could be separated by paper electrophoresis, but

TABLE III

Mobilities of the Main Condensates of Catechol Derivatives with Ethylenediamine

The aqueous solution containing the condensation products corresponding to about 1 μg. of each catechol derivative was placed on the anode side of a paper strip (Whatman No. 1, 2× 32 cm.) moistened with phosphate buffer (pH 8.0, 0.05 *M*) and 1 mA/cm. (300–400 volts) of constant current was applied in a dark. After 5 hours of electrophoresis, the paper strips were examined under ultraviolet light.

Catechol derivatives		Mobility (cm./5 hours)
adrenaline	(main product)	6.5
	(side product)	3.2
noradrenaline		3.2
dopa (without *iso*-butanol extraction)		8.1[a)]
dopamine		5.8[a)]
3,4-dihydroxymandelic acid		3.2
catechol		3.2

a) Tailing of the spot was observed.

the main condensates of noradrenaline, 3,4-dihydroxymandelic acid and catechol, and the side product of adrenaline which fluoresced bluish-green showed the same rate of mobility.

III. The Physico-chemical Properties of the Main Condensates of Catechol Derivatives with Ethylenediamine

Absorption Spectrum—The absorption spectra of aqueous extracts of the main condensates of noradrenaline, 3,4-dihydroxymandelic acid and catechol, and of the side condensate of adrenaline were identical each other and had λ_{max} at 414 mμ, as shown in Fig. 2, whereas those of the main condensation

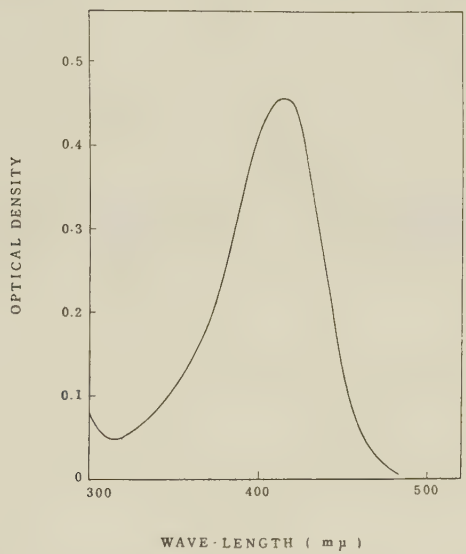

FIG. 2. Absorption spectrum of an aqueous solution of the main condensation product of noradrenaline, 3,4-dihydroxymandelic acid or catechol with ethylenediamine.

products of the other catechol derivatives were different eath other, having λ_{max} as follows: adrenaline at 410 mμ, dopamine at 402 mμ, and dopa at 406 mμ.

Fluorescence Spectrum—The fluorescence spectra of *iso*-butanol extracts of the main condensates of noradrenaline, 3,4-dihydroxymandelic acid and catechol, and that of the side product of adrenaline were identical each other and had λ_{max} at 490 mμ, as shown in Fig. 3, whereas those of the main condensation products of adrenaline and dopamine showed λ_{max} at 550 mμ and 510 mμ, respectively as shown in Fig. 4 and Fig. 5. In the case of dopa, the fluorescence spectrum of the reaction mixture of ethylenediamine with dopa became constant after 2 hours' heating at 50°. The spectrum was shown in Fig. 6 (λ_{max} at 555 mμ).

The main condensation product of adrenaline was stable by the ultraviolet irradiation. But the main condensate of noradrenaline, 3,4-dihydro-

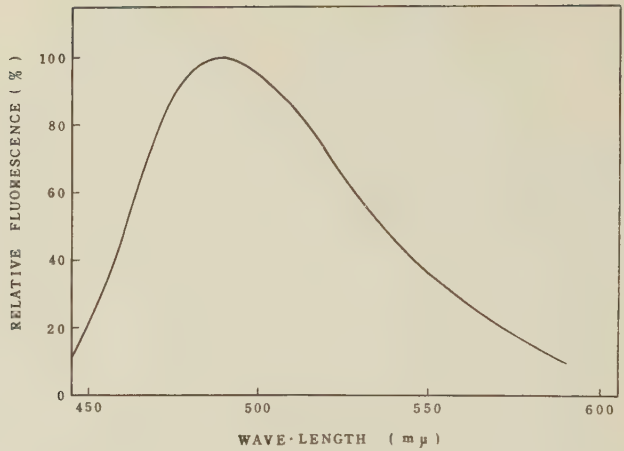

FIG. 3. Fluorescence spectrum of the main condensation product of noradrenaline, 3,4-dihydroxymandelic acid or catechol with ethylenediamine. Samples were dissolved in *iso*-butanol, and were excited by ultraviolet light (365 mμ). The ordinate shows the relative intensity of the fluorescence.

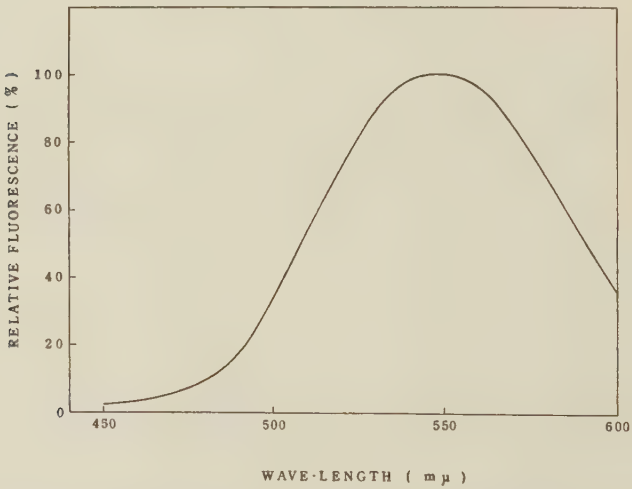

FIG. 4. Fluorescence spectrum of the main condensation product of adrenaline with ethylenediamine. Sample was dissolved in *iso*-butanol, and was excited by ultraviolet light (365 mμ). The ordinate shows the relative intensity of the fluorescence.

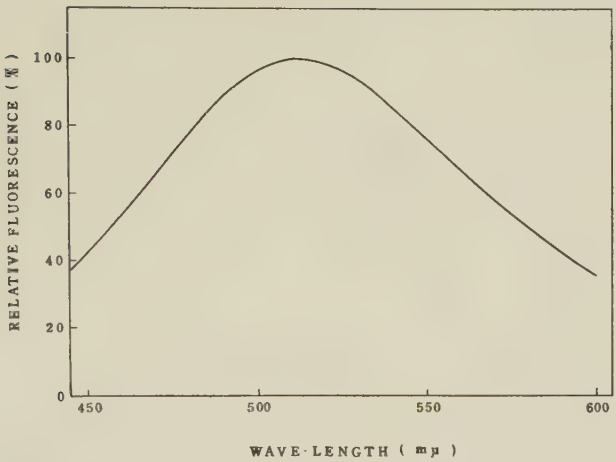

FIG. 5. Fluorescence spectrum of the main condensation product of dopamine with ethylenediamine. Sample was dissolved in *iso*-butanol, and was excited by ultraviolet light (365 mμ). The ordinate shows the relative intensity of the fluorescence.

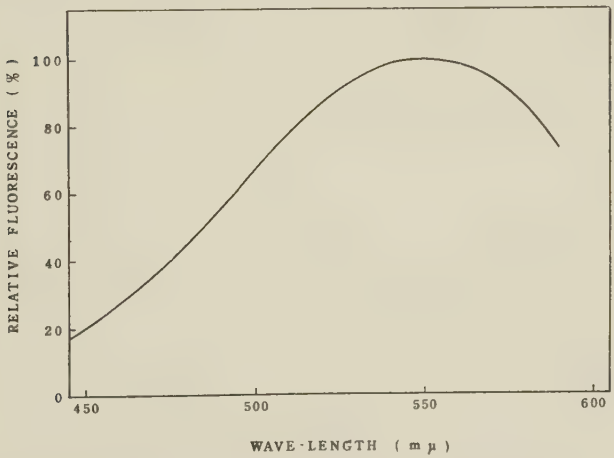

FIG. 6. Fluorescence spectrum of the condensation product of dopa with ethylenediamine. Fluorescence spectrum of the reaction mixture was taken after 2 hours' heating at 50°. The ordinate shows the relative intensity of the fluorescence.

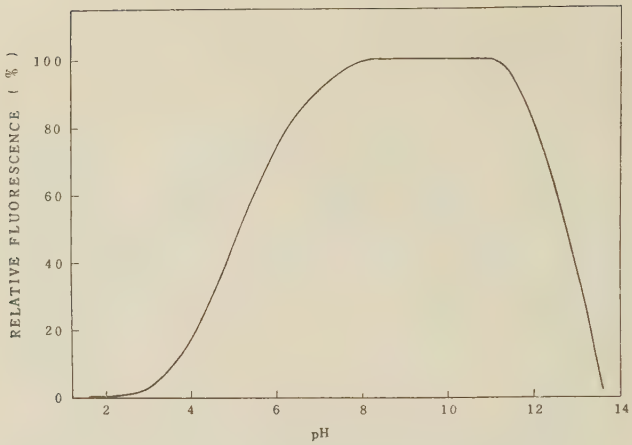

FIG. 7. pH-Fluorescence curve of the main condensation product of noradrenaline, 3,4-dihydroxymandelic acid or catechol, or of the side condensation product of adrenaline. Each condensate was separated on the filter paper and was extracted with water. This sample was dissolved in the buffers of different pH's. The buffer was a mixture of equivolume of 0.1 *M* glycine, 0.1 *M* Na_2HPO_4, 0.1 *M* KH_2PO_4, and the pH was adjusted by adding 0.1 *N* NaOH or 0.1 *N* HCl. For below pH 1, or above pH 13, the aqueous solution of HCl or NaOH was used. The relative fluorescence intensity of the solution at indicated pH, excited by ultraviolet light (365 mμ), was measured at about 20°. The ordinate shows the relative intensity of the fluorescence.

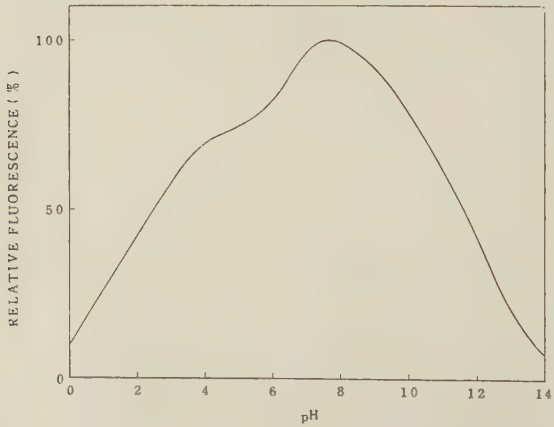

FIG. 8. pH-Fluorescence curve of the main condensation product of adrenaline. The conditions for the estimation was the same as described in Fig. 7. The ordinate shows the relative intensity of the fluorescence.

xymandelic acid or catechol, and the side condensate of adrenaline could be easily photodecomposed by the ultraviolet irradiation. The main condensate of dopa or dopamine was also photodecomposed, but their degrees of decomposition were the same each other, and smaller than the above-mentioned substances.

pH-Fluorescence Curves of the Main Condensation Products of Catechol Derivatives with Ethylenediamine—pH-Fluorescence curves of aqueous extracts of the

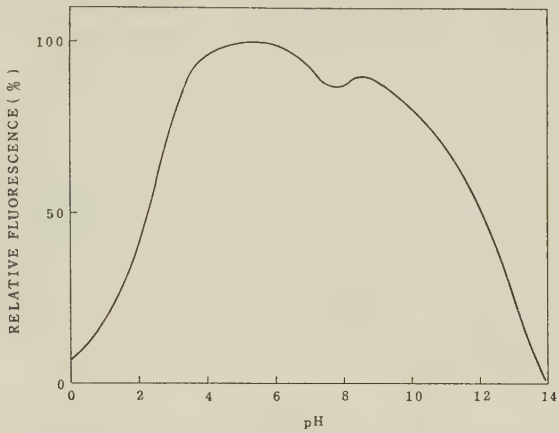

Fig. 9. pH-Fluorescence curve of the main condensation product of dopamine. The conditions for the estimation were the same as described in Fig. 7. The ordinate shows the relative intensity of the fluorescence.

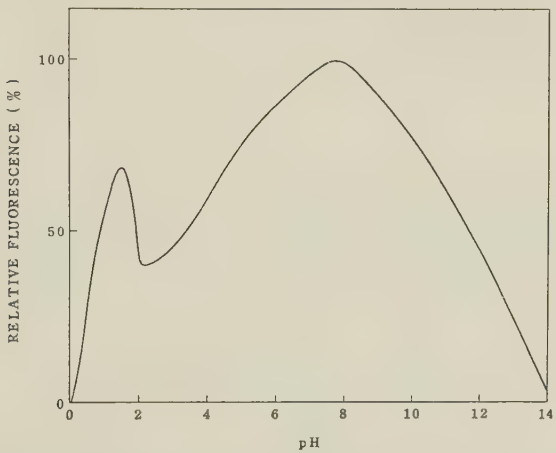

Fig. 10. pH-Fluorescence curve of the main condensation product of dopa. The conditions for the estimation were the same as those described in Fig. 7. The ordinate shows the relative intensity of the fluorescence.

main condensation product of noradrenaline, 3,4-dihydroxymandelic acid or catechol, and of the side condensation product of adrenline were iden-

tical, as shown in Fig. 7, whereas those of the main condensation products of adrenaline, dopamine, and dopa were different each one another as shown in Fig. 8, Fig. 9, and Fig. 10.

DISCUSSION

It was found that the main condensates of ethylenediamine with various catechol derivatives could be analyzed by paper chromatography or by paper electrophoresis. When the adrenaline condensate was tested, two fluorescent spots were observed besides the main condensate. One of them was attributed to the substance produced by the reaction of ethylenediamine dissolved in *iso*-butanol with the substance contained in filter paper. Another bluish-green fluorescent spot was found to have the same R_f value as that of the main condensate of noradrenaline. Their mobilities in paper electrophoresis were identical. The physico-chemical properties of both substances also agreed. These results confirm the identity of the substance appeared in adrenaline condensate and the main condensate of noradrenaline (*9*).

Consequently, it should be noted that adrenaline gives the main condensate of noradrenaline during the condensation reaction with ethylenediamine as its side product.

Further studies carried out in the same way confirmed the identity of the four bluish-green fluorescent substances: the main condensates of noradrenaline, of 3,4-dihydroxymandelic acid, and of catechol, and the side product of adrenaline. So, it was supposed that the side chain of noradrenaline, or of 3,4-dihydroxymandelic acid might be severely destructed during the condensation reaction and form the same condensation product with that of ethylenediamine and catechol. Thus the chemical structure of the main condensation product of noradrenaline or 3,4-dihydroxymandelic acid is considered to be the same as that of catechol, which had been determined to be 1:2:3:4-tetrahydro-1:4:5:8-tetraaza-anthracene by Harley-Mason *et al.* (*7*). These facts suggest that the β-hydroxy group in the side chain might have some relation with the destruction of the side chain during the condensation. Moreover, considering the chemical structure of the main condensation product of adrenaline, dihydro-3-hydroxy-1-methylpyrrolo-[4:5-g]-quinoxaline (*7*), the conjunction of methyl group was supposed to have some protective effect on the destruction of the side chain during the condensation reaction.

The fact that these catechol derivatives mentioned above give the same condensation product should be noted for the specificity of ethylenediamine method in the fluorimetric determination of catechol derivatives in biological materials. Namely, if 3,4-dihydroxymandelic acid or catechol is present in the sample, usual ethylenediamine method for the separation determination of adrenaline and noradrenaline gives high value for noradrenaline.

This case was realized in the recent experiment (*21*). When the

metabolites of L-adrenaline by rat liver mitochondria were analyzed by the use of paper chromatographic separation of the ethylenediamine condensates of the metabolites after alumina purification, the bluish-green fluorescent spot at R_f 0.09 (by solvent 1) was greatly increased comparing with the control. This increase was found to be greater than that expected from the noradrenaline formation in the experiment reported before (*22*). On the other hand, both noradrenaline and 3,4-dihydroxymandelic acid were detected in the metabolite by IRC-50 column chromatography (*23*). So, the increase of the fluorescence of the spot was attributed to the formation of both noradrenaline and 3,4-dihydroxymandelic acid.

Finally, among the results reported in the present paper, the following two facts must be remarked: (i) during the condensation reaction with ethylenediamine, adrenaline gives the main condensate of noradrenaline as its side product, (ii) the main condensate of noradrenaline was identified with that of 3,4-dihydroxymandelic acid or catechol.

Taking these remarks into consideration, ethylenediamine method together with the paper chromatographic or paper electrophoretic techniques, could be successfully applied to various materials which contain very small amount of catechol or its derivatives (0.1–0.5 μg.).

SUMMARY

1. The condensation products of ethylenediamine with various catechol derivatives were studied. The main condensates of adrenaline, noradrenaline, dopamine and dopa could be separated by papar chromatography or by paper electrophoresis. Absorption and fluorescence spectra and pH-fluorescence curves of these substances were different each other. These properties can be used for identification of catechol derivatives.

2. During the condensation reaction with ethylenediamine, adrenaline gives the main condensation product of noradrenaline and ethylenediamine as its side product.

3. The main condensate of noradrenaline was found to be identical with that of 3,4-dihydroxymandelic acid or catechol. This might be important to consider the specificity of ethylenediamine method in the fluorimetric determination of catechol derivatives of biological importance.

Authors' thanks are due to Dr. M. D. Armstrong, the Fels Research Institute, Ohio, who kindly supplied 3,4-dihydroxymandelic acid, and to Dr. T. Kametani, Tohoku University, Sendai, who kindly synthesized dopamine.

REFERENCES

(*1*) Natelson, S., Lugovoy, J. K., and Pincus, J. B., *Arch. Biochem. Biophys.*, **23**, 157 (1949)

(*2*) Weil-Malherbe, H., and Bone, A. D., *Biochem. J.*, **51**, 311 (1952)

(*3*) Weil-Malherbe, H., and Bone, A. D., *Lancet*, **264**, 974 (1953)

(*4*) Burn, G. P., and Field, E. O., *Nature*, **178**, 542 (1956)

(*5*) Elliman, G. L., *Nature*, **181**, 768 (1958)
(*6*) Nadeau, G., and Joly, L. P., *Nature*, **182**, 180 (1958)
(*7*) Harley-Mason, J., and Laird, A. H., *Biochem. J.*, **69**, 59 p (1958)
(*8*) Yagi, K., and Nagatsu, T., *Nature*, **183**, 822 (1959)
(*9*) Yagi, K., Nagatsu, T., and Nagatsu, I., *Nature*, **186**, 310 (1960)
(*10*) Vogt, M., *Brit. J. Pharmacol.*, **7**, 325 (1952)
(*11*) James, W. O., and Kilbey, N., *Nature*, **166**, 67 (1950)
(*12*) Kirshner, N., and Goodall, M., *J. Biol. Chem.*, **226**, 207 (1957)
(*13*) Yagi, K., and Arakawa, T., *Vitamins* (*Kyoto*), **6**, 523 (1953)
(*14*) Erne, K., and Canbäck, T., *J. Pharmac. Pharmacol.*, **7**, 248 (1955)
(*15*) Goldfien, A., and Karter, R., *Science*, **127**, 1292 (1958)
(*16*) Yagi, K., Nagatsu, T., and Nagatsu-Ishibashi, I., *J Biochem.*, (in the press)
(*17*) Weil-Malherbe, H., and Bone, A. D., *Biochem. J.*, **58**, 132 (1954)
(*18*) Gray, I., and Young, J. G., *Clin. Chem.*, **3**, 239 (1957)
(*19*) Yagi, K., Kondo, H., and Sumi, M., *J. Japan. Biochem. Soc.*, **27**, 777 (1956)
(*20*) Yagi, K., Tabata, T., Kotaki, E., and Arakawa, T., *Vitamins* (*Kyoto*), **7**, 878 (1954)
(*21*) Nagatsu, T., *J. Japan. Biochem. Soc.*, **31**, 782 (1959)
(*22*) Yagi, K., and Nagatsu, T., *Nature*, **184**, 1316 (1959)
(*23*) Nagatsu, T., and Yagi, K., *J. Biochem.*, (in the press)

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A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

V. PROTECTIVE EFFECT OF CALCIUM ION ON THE STABILITY OF PROTEASE*

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Like the well known case of trypsin (*1, 2*), it has been recently reported that a few kinds of bacterial proteinase are protected from inactivation by the presence of a small amount of metal ion such as calcium, sodium, zinc, *etc.* (*3–8*).

The present authors also found that *Streptomyces griseus* protease preparation prepared by Duolite S-30 (a non-ion-exchange resin) column treatment showed a remarkable loss of enzyme activity following a salting out procedure with ammonium sulfate. Such a strange phenomenon has never been observed in the previously reported purification process of this enzyme (*9, 10*) in which a cation exchange resin, *e.g.*, Kaken C-1 (SP-3) or Duolite C-10, was used for resin column treatment instead of Duolite S-30. Considering that this anomaly may be caused by a deficiency of metal ion in the enzyme preparation, the present authors substantiated the above remarks by the following experiments which confirmed that the above inactivation of the enzyme was completely prevented by the presence of a small amount of calcium ion in the enzyme solution. The authors also found that the protease was fairly unstable and readily inactivated by dialysis, ion-exchange resin column treatment or heating under a condition in which calcium ion was deficient in the enzyme solution. The protease was also inactivated irreversiblly by mixing it with EDTA. Calcium ion seems to be an essential factor for stabilizing *Streptomyces griseus* protease. The other metal ions, excepting strontium ion, were incapable of serving as a protective factor.

MATERIALS AND METHOSD

Enzyme—The protease preparation mainly used for the present work was prepared by the method shown in Scheme I. This method is almost the same as that previously reported (*10*) with one important exception that Duolite S-30 (a non-ion-exchange resin) is newly adopted in the second resin column treatment instead of Kaken C-1 (SP-3) (an ion-exchange resin) which was used in the previous method (*9, 10*). In the case in

* A large part of the data was published in Japanese in *the Reports of the Institute of Physical and Chemical Research*, **35**, 90, (1959).

which the non-ion-exchange resin is used for resin column treatment, the protease is the only adsorbate on the column but not metal ions. Therefore, the resulting enzyme solution obtained by eluting the enzyme from the column contains a very small amount of calcium ion and the lyophilized enzyme preparation prepared from the above enzyme solution is also in the state of deficiency of calcium ion. The Kaken Phenol-type resin (a non-ion-exchange resin) is also available for preparing the calcium deficient enzyme solution. In the case in which ion-exchange resin (*e. g.*, Kaken C-1 (SP-3) or Duolite C-10) is employed for the column treatment, calcium ion is adsorbed on and eluted from the column together with the protease and accordingly the resulting enzyme solution contains sufficient amounts of calcium ion to prevent the inactivation of the enzyme.

SCHEME I

Method for Preparing Calcium-deficient Preparation of Streptomyces griseus Protease

Preparation	*Purification process*
Culture broth filtrate	
	Adsorptive exclusion of streptomycin and coloring matter by resin column treatment with Kaken C-1 (SP-7) resin
Effluent from Kaken C-1 (SP-7) resin column	
	Adsorption and elution of enzyme by resin column treatment with Duolite S-30 resin
Eluate from Duolite S-30 resin column	
	Fractional precipitation with acetone (50–66 per cent of acetone concentration)
Acetone precipitate	
	Dissolve in distilled water, and lyophilized
Lyophilized preparation (calcium deficient preparation)	

For the experiments of inactivation of the protease by dialysis or EDTA treatment, the highly purified lyophilized enzyme preparation prepared by the previously reported method (*11*) was used.

Assay—The proteinase activity was measured by the casein-Folin color method described in the preceding paper (*9*). The activity assayed by this method represents the reaction volocity producing a trichloroacetic acid soluble digestion product from casein and is indicated by *PU* values as previously described (*9*).

Nitrogen concentration was also determined by the previously reported method (*9*) and is represented by the *PN* values.

Preparation of Resin—Na-form resin: Kaken C-1 (SP-3) or Duolite C-10 was adopted for the present purpose. After washing the resin (60–80 mesh) several times with $N/2$ NaOH, water, $N/2$ HCl and water alternately, the resin is suspended in ten volumes of $N/2$ NaOH and kept in it for 24 hours. The resin is then transferred into a column

and washed by passing five volumes of $N/2$ NaOH followed by distilled water through the column until the pH of the effluent becomes neutral.

Ca-form resin: After suspending the above mentioned Na-form resin in ten volumes of $M/2$ $CaCl_2$ solution overnight, the resulting resin was transferred into a column and washed by passing $M/2$ $CaCl_2$ solution followed by a small amount of distilled water through the column.

Buffered-form resin: After suspending the above Na-form resin in ten volumes of $M/15$ sodium phosphate buffer (pH 6.0) overnight, the resulting resin was transferred into a column and washed by passing five volumes of the same buffer through the column.

Resin Column Treatment—Each 3 g. of resin prepared as above was put into a column, 12 mm. in diameter. Through the column, about 50 ml. of the enzyme solution was passed at the rate of 0.3 ml. per minute. Adsorption capacity of the resin was calculated as the difference in the total activity between the original enzyme solution and the effluent. After washing the column with a small amount of distilled water so as to drive out the remaining enzyme solution, the enzyme adsorbed on the column was then eluted by passing 50 ml. of $M/2$ sodium borate buffer (pH 9.2, containing $M/2$ KCl) through the column at the rate of 0.15 ml. per minute. The effluent was immediately neutralized and assayed.

RESULTS AND DISCUSSION

Protective Effect of Calcium Ion in Salting Out Procedure—The calcium deficient protease preparation prepared as described in Scheme I. was dissolved in

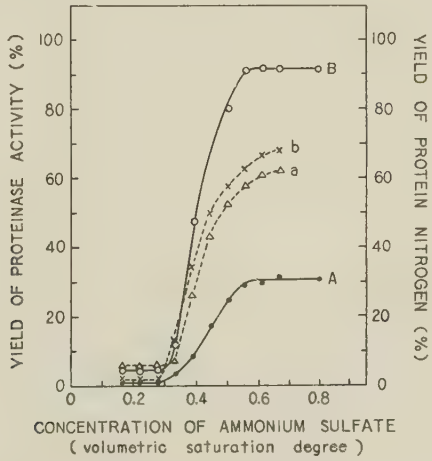

Fig. 1. Protective effect of calcium ion in salting out procedure.

The enzyme solution containing 233×10^{-3} *PU*/ml. of proteinase activity was mixed with various amounts of ammonium sulfate and the mixture was kept at room temperature for 3 hours.

(A), (B) indicate proteinase activity and (a), (b) indicate protein-N content in the resulting precipitate. In the case of (B), (b) 0.01 M $CaCl_2$ is present in the original enzyme solution but not in the case of (A), (a).

distilled water and mixed with ammonium sulfate at a gradient concentration of the salt. As shown in Fig. 1, the yield of the enzyme activity in the resulting precipitates reaches a plateau of 30–40 per cent recovery as compared with the original activity (Curve-A), whereas the yield of the Protein-N in the same precipitates comes up to 60 per cent or more (Curve-a). This apparently shows that inactivation of the enzyme is invited by the salting out procedure. On the other hand, when a small amount of calcium salt is previously added in the enzyme solution before the salting out procedure, more than 90 per cent of the enzyme activity and about 60 per cent of the protein-N are recovered in the resulting precipitates obtained at a higher concentration of ammonium sulfate (Curve-B and b, respectively). This shows a fair protective effect of calcium ion on the stability of the protease activity.

Table I

Influence of Calcium Ion Concentration in Salting Out Procedure[1)]

Concentration of calcium ion added (*M*)	Proteinase activity contained in the precipitate (*PU*)	Yield (%)
0.1	996.5×10^{-3}	103
0.04	972.8 ,,	100
0.02	997.5 ,,	103
0.01	903.7 ,,	93

1) To the enzyme solution containing 969.8×10^{-3} *PU* of proteinase activity, 40 g./dl. of ammonium sulfate was added and the mixture was kept at room temperature for 2 hours.

Table II

Protective Effects of Various Metal Ions in Salting Out Procedure[1)]

Kind of metal ion added	Concentration (*M*)	Proteinase activity contained in the precipitate (*PU*)	Yield (%)
$Na(CH_3COO)$	0.1	201.0×10^{-3}	85
$Ca(CH_3COO)_2$	0.1	223.7 ,,	94
$Zn(CH_3COO)_2$	0.1	122.5 ,,	52
$SrCl_2$	0.1	227.5 ,,	96

1) To the enzyme solution containing 237.0×10^{-3} *PU* of proteinase activity and 0.1 *M* of metal ion, 40 g./dl. of ammonium sulfate was added and the mixture was kept at 4° for 1 hour.

The effective concentration of calcium ion seems to be more than 0.01 *M* (Table I). Strontium ion was also proved to be effective but not sodium ion or zinc ion (Table II).

Protective Effect of Calcium Ion in Dialysis—A highly purified protease preparation was dissolved in 0.01 *M* calcium acetate solution. Each 1 ml. of the enzyme solution was put in a small cellophane tube and dialyzed against distilled water or several kinds of salt solution for 24 hours at 4° and at 20°. After the dialysis, the residual enzyme activities in the tubes were compared with each other in order to examine the protective effect of mineral ion during dialysis. As shown in Table III, it is apparent that calcium ion is remarkably effective but not the other metal ions such as sodium, potassium, ammonium, magnesium, zinc, barium, *etc.*

Table III

Protective Effect of Various Metal Ions in Dialysis[1)]

Temperature in dialysis	Outside solution used for dialysis	Residual proteinase activity (*PU*)	Yield (%)
4°	distilled water	76.5×10^{-3}	35
	0.01 *M* $Ca(CH_3COO)_2$	216.3 ,,	99
20°	distilled water	23.2 ,,	11
	well water	156.5 ,,	72
	0.02 *M* NaCl	92.8 ,,	43
	0.02 *M* $Na(CH_3COO)$	100.2 ,,	46
	0.02 *M* KCl	82.0 ,,	38
	0.02 *M* NH_4Cl	75.1 ,,	34
	0.01 *M* $CaCl_2$	163.0 ,,	75
	0.01 *M* $Ca(CH_3COO)_2$	162.5 ,,	74
	0.01 *M* $MgCl_2$	51.4 ,,	24
	0.01 *M* $ZnCl_2$	95.8 ,,	44
	0.01 *M* $BaCl_2$	41.5 ,,	19
	0.01 *M* $MnCl_2$	77.5 ,,	36
	0.01 *M* $CoCl_2$	26.2 ,,	12

1) The enzyme solution containing 218.3×10^{-3} *PU* of enzyme activity was dialyzed against various solutions for 24 hours at 4° and at 20°.

Protective Effect of Calcium Ion in Heating—The protease was also found to be protected from heat inactivation by the presence of calcium ion. As shown in Fig. 2, when the calcium deficient enzyme solution is heated at 60° for 10–20 minutes, 70–80 per cent of the proteinase activity contained in the original enzyme solution is destroyed by heating. On the other hand, when a small amount of calcium ion is previously added to the above solution, the inactivation of the enzyme is no more than 20–30 per

cent. Strontium ion also proved to be effective, but not sodium ion or magnesium ion.

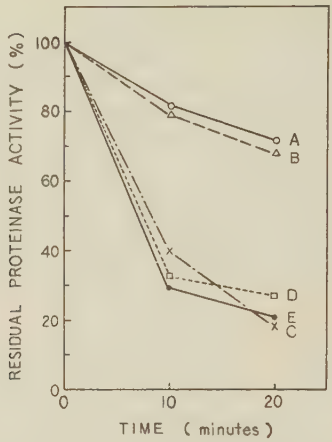

FIG. 2. Influence of various metal ions upon the heat-stability of *Streptomyces griseus* protease.

The enzyme solution containing 0.95×10^{-4} *PU*/ml. of proteinase activity was heated at 60° for 10–20 minutes.

In the case of (A) 0.01 *M* $CaCl_2$, (B) 0.01 *M* $SrCl_2$, (C) 0.01 *M* $MgCl_2$, (D) 0.01 *M* NaCl was added into the enzyme solution but not in the case of (E), which is the control.

Protective Effect of Calcium Ion in Ion Exchange Resin Column Treatment— When the calcium deficient enzyme solution prepared by Duolite S-30 resin column treatment was treated once more by Kaken C-1 (SP-3) or Duolite C-10 resin buffered at pH 6.0, a marked inactivation of the protease was observed. That is, as shown in Table IV, the protein-N adsorbed on the ion-exchange resin column is recovered almost completely in the eluate but not the proteinase activity. On the other hand, when a small amount of calcium ion is previously added to the enzyme solution before charged into the ion-exchange resin column, a good yield of the enzyme activity is detected in the eluate showing the fair protective effect of calcium ion in the resin column treatment. The effective concentration of calcium ion was found to be more than 0.01*M*.

On the basis of the data that almost all members of the functional group of the above resins buffered at pH 6.0 are present in a state of Na-form and that the velocity of exchange reaction of sodium ion for calcium ion is extremely rapid, the authors considered that the ion-exchange resins prepared at Ca-form should be also available without the previous addition of calcium ion into the enzyme solution. As shown in Table V, a good

TABLE IV

Protective Effect of Calcium Ion on the Stability of Streptomyces griseus Protease in the Ion-Exchange Resin Column Treatment[1)]

Kind of Resin		Kaken C-1 (SP-3)		Duolite C-10	
Form of Functional Group of Resin		Buffered at pH 6.0	Buffered at pH 6.0	Buffered at pH 6.0	Buffered at pH 6.0
Concentration of Calcium Ion added		not added	0.05 *M*	not added	0.05 *M*
Original Enzyme Solution	*PU*	1,864	1,864	1,864	1,864
	PN	93	93	93	93
Effluent Solution passed through the Column	*PU*	73	25	584	578
	PN	7	3	29	28
Apparent Amount of Enzyme adsorbed on the Column	*PU*	1,791	1,839	1,280	1,286
	PN	86	90	64	65
Eluate from the Column	*PU*	360	1,166	458	956
	PN	78	80	63	64
Elution Ratio (The Eluate/The Adsorbate)		20%	63%	36%	74%

1) Each 3 g. of the resin was put into a column, 12 mm. in diameter. Through the column, about 50 ml. of the enzyme solution was passed. Amount of the enzyme adsorbed on the column was calculated as the defference between the original solution and the effluent. The adsorbed enzyme was eluted from the column by passing 50 ml. of sodium borate buffer (pH 9.2).

The *PU* indicates the proteinase activity assayed by the casein-Folin color method and the *PN* indicates the protein-N concentration assayed by the Folin color method.

TABLE V

Influence of the Functional Group Form of Resin upon the Elution Ratio in the Resin Column Treatment[1)]

Eorm of Functional Group of Kaken C-1 (SP-3)		Ca-form		Na-form		Buffered-from (pH 6.0)		Ca-form		Na-form		Buffered-form (pH 6.0)	
Concentration of Calcium Ion added		not added		not added		not added		0.05 *M*		0.05 *M*		0.05 *M*	
Original Enzyme Solution	*PU*	2,500		2,500		2,500		2,500		2,500		2,500	
	PN		130		130		130		130		130		130
Ffluent Solution passed through the Column	*PU*	272		628		362		247		284		778	
	PN		29		49		45		31		30		57
Apparent Amount of Enzyme adsorbed on the Column	*PU*	2,282		1,872		2,138		2,253		2,216		1,722	
	PN		101		81		85		99		100		73
Eluate from the Column	*PU*	1,609		843		740		1,531		1,487		1,071	
	PN		77		51		67		69		66		58
Elution Ratio (The Eluate/The Adsorbate)		72%		45%		34%		68%		67%		63%	

1) The resin column treatment was carried out similarly as described in Table IV.

PU: Proteinase activity assayed by the casein-Folin color method, *PN*: Protein-N concentration assayed by the Folin color method.

yield of the enzyme activity resulted by the use of the Ca-form resin without previous addition of calcium ion into the enzyme solution but not by the use of the Na-form resin, as was expected.

The above information seems to indicate that calcium ion is not only the stabilizing factor of the protease but also the medium of enzyme-resin complex formation on the resin column treatment. On the hypothesis that calcium ion acts as a special medium, a model of enzyme-resin complex formation is presented in Fig. 3.

(A) In the case of Ca-ion is deficient in the enzyme solution and Na-form resin is used.

(B) In the case of Ca-ion is present in the enzyme solution or Ca-form resin is used.

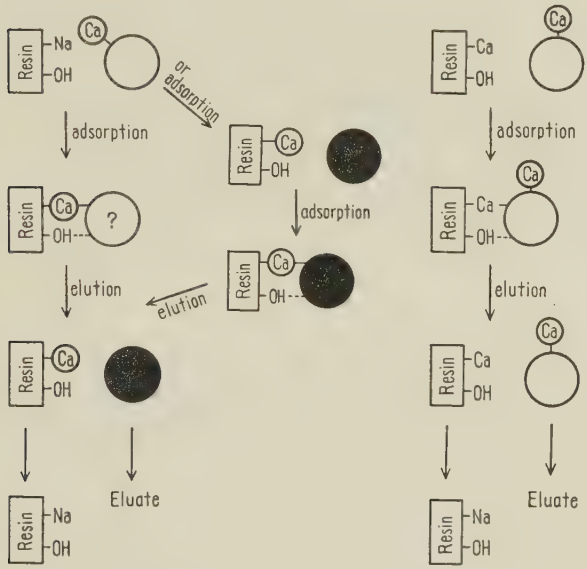

Fig. 3. A model of enzyme-resin complex formation in resin column treatment in the presence of, and in the absence of calcium ion.

○——Enzyme protein (native form)

●——Enzyme protein (denatured form)

Inactivation of the Protease by Addition of EDTA—The protease was found to be inactivated irreversiblly by mixing it with EDTA (Fig. 4). It seems that an active configuration of the protease can be maintained and the denaturation of the enzyme protein can be prevented by combining the enzyme with calcium ion.

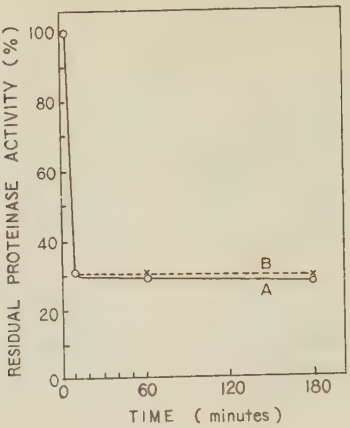

FIG. 4. Inactivation of *Streptomyces griseus* protease by addition of EDTA.

The enzyme solution containing 4.0×10^{-3} *PU*/ml. of proteinase activity was mixed with $5 \times 10^{-4} M$ of EDTA and the mixtures was kept at 4°.

In the case of (B) $10^{-3} M$ of calcium ion was added into the mixture after 10 minutes reaction but not in the case of (A).

SUMMARY

Calcium ion acts as an essential factor for stabilizing *Streptmyces griseus* protease. Under conditions in which calcium ion is deficient, the enzyme was readily inactivated by the procedures of dialysis, salting out, ion-exchange resin column treatment, or heating. It was found, however, that the enzyme was highly protected from inactivation by the presence of a small amount of calcium ion during the above mentioned procedures. The enzyme was also inactivated irreversiblly by mixing it with EDTA. It seems that the active configuration of the enzyme can be maintained and denaturation prevented by combining the enzyme with calcium ion. Strontium ion also proved capable of serving as a protective ion, but not other metal ions.

In connection with the above investigations, it was found that the enzyme was adsorbed on and eluted from the cation exchange resin column in which the functional group of resin was prepared in the state of calcium form, in a good yield. This information seems to indicate that calcium ion is not only the stabilizing factor of the enzyme but also the medium of enzyme-resin complex formation in resin column treatment.

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REFERENCES

(*1*) Gorini, L., *Biochim. et Biophys. Acta*, **7**, 318 (1951)
(*2*) Bier, M. and Nord, F. F., *Arch. Biochem. Biophys.*, **33**, 320 (1951)
(*3*) Hagihara, B., *Ann. Rep. Sci. Works, Osaka Univ.*, **2**, 35 (1954)
(*4*) Matsubara, H., Hagihara, B., Nakai, T., Yonetani, T., and Okunuki, K., *J. Biochem.*, **45**, 251 (1958)
(*5*) Hamaguchi, Y. and Takahashi, K., *J. Japan. Biochem. Soc.*, **29**, 780 (1958)
(*6*) Fukumoto, J., Yamamoto, T., Ichikawa, K., and Sakube, M., *J. Agr. Chem. Soc. Japan*, **32**, 233 (1958)
(*7*) Fukumoto, J., Yamamoto, T., Tsuru, D., and Ichikawa, K., *Symposium on Enzyme Chem.*, **13**, 193 (1958)
(*8*) Morihara, K., *Bull. Agr. Chem. Soc.*, **21**, 12 (1957)
(*9*) Nomoto, M. and Narahashi, Y., *J. Biochem.*, **46**, 653 (1959); *Reports of the Institute of Physical and Chemical Research*, **34**, 381 (1958)
(*10*) Nomoto, M. and Narahashi, Y., *J. Biochem.*, **46**, 839 (1959); *Reports of the Institute of Physical and Chemical Research*, **34**, 393 (1958)
(*11*) Nomoto, M. and Narahashi, Y., *J. Biochem.*, **46**, 1481 (1959); *Reports of the Institute of Physical and Chemical Research*, **34**, 399 (1958)

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CHROMATOGRAPHIC STUDIES ON IODINATED BOVINE PLASMA ALBUMIN

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The iodinated albumin has been extensively used for the studies of albumin metabolism and immunochemical reaction. However, there was no investigation on the reactivity of iodine with albumin subfractions except by Fahey (*1, 2*). The authors studied the reactivity of iodine with Armour's bovine plasma albumin subfractions with the aid of hydroxylapatite column, radioactive iodine (I^{131}) and paperchromatography.

The radioiodinated bovine plasma albumin was prepared by the following procedure (*3–6*). An aliquots of 4.40 ml. of carrier free iodide containing 1 mc. of Na I^{131} was added to 0.40 ml. of 0.039 *N* iodine in 0.05 *M* KI. Three tenth per cent bovine plasma albumin solution was prepared by using various buffer solutions, such as, 0.1 *M* of sodium phosphate buffer pH 7.50, 0.05 *M* borate buffer, pH 7.68, 8.89 and 0.05 *M* bicarbonate buffer, pH 9.65 and 10.65. The protein solution and iodinating reagent were immersed in ice-water. Iodinating reagent was added dropwise with stirring to bovine plasma albumin solution and the mixture was kept in ice-water for 60 minutes with (method A) or without stirring (method B). Thereafter, excess sodium thiosulfate was added and the albumin solution was dialysed for 48 hours at 3° with several changes of distilled water. For chromatographic analysis, pH of the dialysed solution was adjusted to 6.80 by 0.02 *M* sodium phosphate buffer. The bound iodine was measured as follows. Precipitates were produced by adding 5.0 ml. of 10 per cent TCA to 2.0 ml. of protein solution, while 2.0 ml. of 0.5 per cent bovine plasma albumin solution was added as carrier prior to the precipitation. The precititates were washed twice with 5 per cent TCA and then dissolved in dilute sodium hydroxide solution and their radioactivity was measured by using G. M. counter (*4*). Assuming a molecular weight of 65,000 for bovine plasma albumin, number of moles of iodine atom per mole of protein was calculated. Enzymic hydrolysis of iodinated albumin and paperchromatography (solvent ; *n*-butanol : acetic acid : water, 78 : 5 : 17 by volume ratio) of iodinated amino acids were carried out according to Roches' report (*7*). Column chromatography was carried out at room temperature by using hydroxylapatite column (1.5 cm. × 12 cm.) which was developed by Tiselius (*8*).

The present authors used very coarse hydroxylapatite which was prepared by the authors' method (*2*). The elution rate was about 30~35 ml. per hour and the effluent was fractionated into 3.5 ml. fraction by fraction collector. Three subfractions were obtained by successive elution with 0.07 *M*, 0.11 *M* 0.40 *M* of sodium phosphate buffer, pH 6.8, respectively. The protein content was analysed by the extinction at 280 mμ in 1 cm. cell in Beckman DU spectrophotometer. I^{131} was measured by G. M. counter after drying 2.0 ml. of each fraction at 50° to uniform film.

In the case of Fig. 1, iodination was carried out by method A at pH

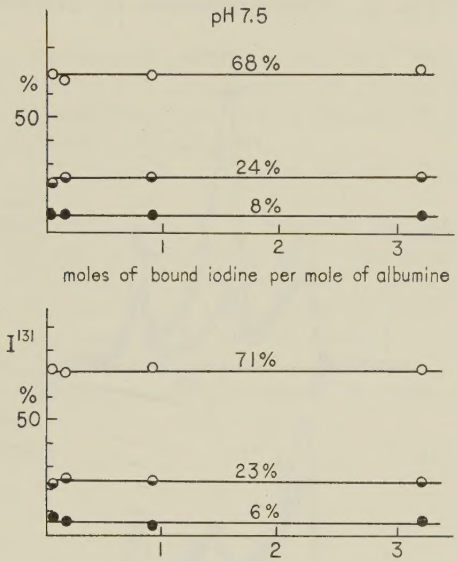

Fig. 1. The upper diagram shows the percentages of three subfractions eluted with 0.07 *M* (○), 0.11 *M* (◒) and 0.41 *M* (●) of sodium phosphate buffer, pH 6.8, against numbers of bound iodine (numbers of moles of iodine atom per mole of bovine plasma albumin). The percentages of three subfractions of uniodinated albumin are 67, 25 and 8 per cent, respectively. The lower diagram shows the percentages of bound I^{131} of three subfractions against numbers of bound iodine.

7.5 (0.1 *M* sodium phosphate buffer). Column chromatograms of iodinated albumin were consistent with those of untreated albumin (ratio of three subfractions of untreated albumin, 67 : 25 : 8). Also, there was no difference in percentages of bound I^{131} of three subfractions among iodinated albumins. Therefore, chromatographic behavior of radioiodinated albumin did not differ from that of albumin with hydroxylapatite column. These results were inconsistent with those of Fahey obtained with DEAH-cellulose (*1*, *2*)*.

* The chromatographic analysis of iodinated albumins illustrated in Fig. 1 and in the upper part of Fig. 2 was carried out by using DEAE-cellulose column (1.5 cm × 1.6 cm, 0.6 m equivalents per dry gram of adsorbent, continuous gradient elution (*1*, *2*)). The results were almost similar to those of hydroxylapatite column. DEAE-cellulose was kindly prepared by Dr. S. Iwanaga (Department of Medicine, Faculty of Medicine, Kyoto University). Finer results will be published in this journal.

When iodination was carried out at pH 9.65 or 10.65 by method B, the greatest radioactivity was found in the second subfraction eluted by 0.11 *M* sodium phosphate buffer, pH 6.8 as shown in the upper part of Fig. 2*. The bound ratio-iodine could not be extracted by *n*-butanol saturated with 0.1 *N* HCl. The high reactivity of second subfraction may be attributable to a specific molecular structure of the protein in the second subfraction as in the cases of its reactivity with L-thyroxine (*10*) as well as its heat stability (*11*). When iodination was carried out by method A, no such difference in radioactivity was observed among three subfractions as shown in the lower part of Fig. 2. The mechanism by which the great difference

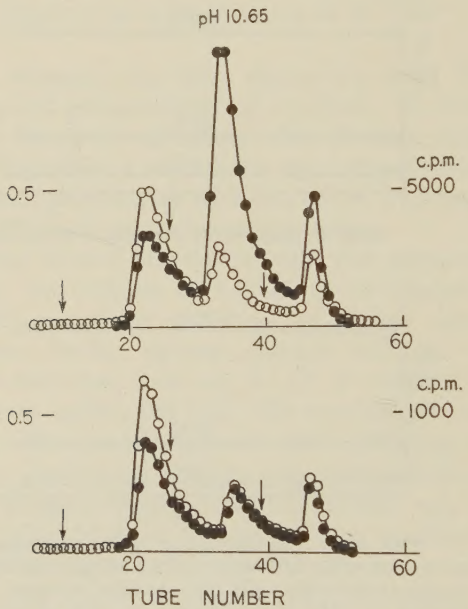

FIG. 2. The left and right ordinates show protein content (○) and radioactivity (●), respectively. The abscissa shows tube numbers of fraction collector. The upper and lower diagrams show the results obtained at pH 10.65 by methods B and A, respectively. The first, second and third arrows indicate 0.07 *M*, 0.11 *M* and 0.40 *M* of sodium phosphate buffer, pH 6.8, respectively.

appears between samples prepared by methods A and B at pH 9.65 and 10.65, will be not clear until more information is available on the iodination reaction.

It was found by paperchromatographic analysis, that upon enzymic hydrolysis, most of the bound iodine (I^{131}) of iodinated albumin illustrated in Fig. 1 was found in tyrosyl residues as monoiodotyrosine and a smaller amount of iodine in thyronine derivatives and diiodotyrosine. On the other hand, it was found that most of the bound iodine (I^{131}) of iodinated albumin

* See the footnote in the page 465.

illustrated in the upper part of Fig. 2 was found in thryonine derivatives, diiodotyrosine and monoidotyrosine.

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REFERENCES

(*1*) Fahey, J. L., and Steinfeld, J. L., *Proc. Soc. Exptl. Biol. Med.*, **97**, 281 (1958)
(*2*) Fahey, J. L., McCoy, P. F., and Goulian, M., *J. Clin. Invest.*, **37**, 272 (1958)
(*3*) Melcher, L. R., and Masouredis, S. P., *J. Immunol.*, **67**, 393 (1951)
(*4*) Cohen, S., *J. Immunol.*, **67**, 339 (1951)
(*5*) Masouredis, S. P., Melcher, L. R., and Koblick, D. C., *J. Immunol.*, **66**, 297 (1951)
(*6*) Hughes, W. L., and Straessle R., *J. Am. Chem. Soc.*, **72**, 452 (1950)
(*7*) Roche, J., Lissitzky, S., and Michel, R., "Methods of Biochemical Analysis," Interscience Publishers, Inc., New York, vol. **1**, 244 (1954)
(*8*) Tiselius, A., Hjertén, S., and Levin, Ö., *Arch. Biochem. Biophys.*, **64**, 217 (1956)
(*9*) Sogami, M., and Takeo, Y., *J. Biochem.*, in press
(*10*) Sogami, M., *J. Biochem.*, **45**, 461 (1958)
(*11*) Sogami, M., and Takemoto, S., *J. Biochem.*, **46**, 1395 (1959)